Etude des acteurs et des interactions entre les voies de recombinaison chez Arabidopsis thaliana

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ÉTUDE DES ACTEURS ET DES INTERACTIONS ENTRE LES VOIES DE RECOMBINAISON CHEZ *ARABIDOPSIS THALIANA*

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Résumé

La réparation des cassures double brin (CDB) de l'ADN par recombinaison est essentielle au maintien de l'intégrité du génome de tous les être vivants. Ce processus doit cependant être finement régulé puisque la recombinaison peut générer des mutations ou des réarrangements chromosomiques, parfois extrêmement délétères pour la cellule. Les CDB peuvent être réparées par deux mécanismes : la recombinaison non homologue (ou jonction des extrémités d'ADN) ou la recombinaison homologue (impliquant une homologie de séquence entre les molécules recombinantes). Dans les cellules somatiques, les deux voies principales de recombinaison homologue (RH) sont la voie *Synthesis Dependent Strand Annealing* (SDSA) dépendante de la recombinase RAD51 et la voie *Single Strand Annealing* (SSA) indépendante de RAD51.

Nos résultats ont d'abord mis en évidence un rôle inattendu de XRCC2, RAD51B et RAD51D - trois paralogues de RAD51 - dans la voie SSA. Nous avons confirmé que la fonction de la protéine XRCC2 dans la voie SSA ne dépend pas de RAD51, ce qui démontre que certains paralogues de RAD51 ont acquis des fonctions indépendantes de la recombinase. La différence de sévérité des phénotypes des mutants individuels ainsi que les analyses d'épistasie menées sur le double et le triple mutant suggèrent des fonctions individuelles de ces protéines au cours du SSA. Nous proposons qu'elles facilitent l'étape d'hybridation des deux séquences complémentaires situées de part et d'autre de la cassure, bien que ceci reste à confirmer par des études *in vitro*.

L'étude des fonctions de l'hétérodimère XPF-ERCC1 - un complexe impliqué dans le clivage des extrémités d'ADN non homologues au cours des voies de RH - a révélé un rôle inhibiteur de ce complexe sur la voie SDSA. Cette action est dépendante de son activité endonucléasique et serait liée au clivage des longues extrémités 3' sortantes réalisant l'invasion d'un duplex d'ADN homologue, l'étape initiale de la voie SDSA. Notre étude a de plus confirmé que le rôle du complexe dépend de la longueur des extrémités non homologues chez *Arabidopsis*, comme chez les mammifères et la levure. Bien que le complexe XPF-ERCC1 soit essentiel au clivage des longues extrémités d'ADN non homologue, il n'est pas requis à l'élimination des courtes extrémités au cours de la RH.

Abstract

The repair of DNA double-strand breaks (DSB) by recombination is essential for the maintenance of genome integrity of all living organisms. However, recombination must be finely regulated as it can generate mutations or chromosomal rearrangements, sometimes extremely deleterious to the cell. DSB can be repaired by two classes of recombination mechanism: non-homologous recombination (or DNA End Joining) or homologous recombination (implicating DNA sequence homology between the recombining molecules). In somatic cells, the two main pathways of homologous recombination (HR) are RAD51-dependent Synthesis Dependent Strand Annealing (SDSA) and RAD51-independent Single Strand Annealing (SSA).

Our results have demonstrated an unexpected role of XRCC2, RAD51B and RAD51D - three RAD51 paralogues - in the SSA pathway. We confirmed that the function of XRCC2 in SSA does not depend upon RAD51, thus demonstrating that some RAD51 paralogues have acquired RAD51 recombinase-independent functions. The different severities of individual mutant phenotypes and epistasis analyses carried out on the double and triple mutants suggest individual functions of these proteins in SSA recombination. We propose that they facilitate hybridization of the two complementary sequences located on both sides of the break, although this remains to be confirmed by *in vitro* experiments.

Study of the roles of XPF-ERCC1 - a complex involved in the cleavage of non-homologous DNA ends during HR - revealed an inhibitory role of this complex on the SDSA pathway. This is dependent on its endonuclease activity and is probably due to the cleavage of long 3' ends performing the homologous DNA duplex invasion, the initial step of the SDSA pathway. Our analyses also confirmed that the role of the complex depends on the length of the non-homologous ends, as seen in mammals and yeasts. Although XPF-ERCC1 is essential for the cleavage of long non-homologous DNA ends, it is not required for the elimination of short ends during HR.

Mots-clés

Réparation de l'ADN, Cassure double brin, Recombinaison homologue, Single Strand Annealing, Synthesis Dependent Strand Annealing, XRCC2, RAD51B, RAD51C, XPF-ERCC1, *Arabidopsis thaliana*

Table des matières

Liste des abréviations

ABL : Abelson leukemia virus tyrosine kinase
ADN : Acide désoxyribonucléique
ADNdb : ADN double brin
ADNg : ADN génomique
ADNsb : ADN simple brin
aNHEJ : alternative NHEJ
AP : Apurinique ou apyrimidique
ARN : Acide ribonucléique
ARNm : Acide ribonucléique messager
ATM : Ataxia telangiectasia mutated
ATP : Adénosine triphosphate
BARD : BRCA1-associated ring domain
BCDX2 : RAD51B-RAD51C-RAD51D-XRCC2
BCR : Breakpoint cluster region
BER : Base excision repair
BIR : Break-induced replication
BLM : Bloom
bNHEJ : backup NHEJ
Boucle D : boucle de déplacement
BRCA : Breast cancer
CDB : Cassures double brin
CENH : Centromeric histone
CFP : Cyan fluorescent protein
CG : Conversion génique
CHO : Chinese hamster ovary
C-NHEJ : Canonical NHEJ
CO : Crossing-over
CSB : Cassure simple brin
CSM : Chromosome segregation in meiosis
CtIP : CtBP-interacting protein
CX3 : RAD51C-XRCC3
dJH : double jonction de Holliday
DMC : Disrupted meiotic cDNA
DNA-PKcs : DNA-dependent protein kinase catalytic subunit
DNL : DNA ligase
dRP : désoxyribose phosphate
DSBR : Double strand break repair
EME : Essential meiotic structure-specific endonuclease
ERCC : Excision repair cross complementing
ES : Embryonic stem
EXO : Exonuclease
FANCD : Fanconi anemia group D
GFP : Green fluorescent protein
H2B : Histone 2B
HML : Hidden MAT left
HMR : Hidden MAT right
HO : Homothallic switching endonuclease
HU : Hydroxyurea
ICL : Interstrand crosslink
IG : Immunoglobuline
IR: Ionizing radiation
JH : Jonction de Holliday
kb : kilobase
kDa : kiloDalton
KO : Knockout
LIF : Ligase interacting factor
MAT : Mating type
Mb : Mégabase
MEC : Mitosis entry checkpoint
MMC : Mitomycine C
MMEJ : Microhomology-mediated end joining
MMR : Mismatch repair
MMS : Méthyl méthane sulfonate
MRN : MRE11-RAD50-NBS1
MRX : MRE11-RAD50-XRS2
MSH : MutS homolog
MUS : Mutagen sensitive
NCO : Non crossing-over
NER : Nucleotide excision repair
NHEJ : Non homologous end joining
NPF : Nucleoprotein filament
P35S : Promoteur 35S

PALB : Partner and localizer of BRCA
PARP : Poly(ADP-ribose) polymerase
pb : paire de bases
PCR : Polymerase chain reaction
PEG : Polyéthylène glycol
PNKP : Polynucleotide kinase /phosphatase
PSY : Platinum sensitivity
RAG : Recombination Activating Gene
RDH : RAD homologue
RDL : RAD51D-like protein
RFS : Rad55 suppressor
RH : Recombinaison homologue
RHP : Rad homolog S. pombe
RLP : RecA-like protein
RMI : RecQ-mediated genome instability
ROS : Reactive oxygen species
RPA : Replication protein A
RSS : Recombination signal sequence
RT : Reverse transcription
SAE : Sporulation in the Absence of SPO Eleven
SAW : Single-strand annealing weakened
SDSA : Synthesis-dependent strand-annealing
SGS : Slow Growth Suppressor
SHU : Suppressor of sgs1 hydroxyurea sensitivity
SLX : Synthetic lethal of unknown function
SPN : Spindle
SPO : Sporulation
SSB : Single-stranded DNA-binding
SSA : Single strand annealing
SSC : Side scatter
SWS : SWIM domain-containing and Srs2-interacting protein
SWSAP : SWS associated protein
T35S : Terminateur 35S
TdT : Terminal deoxynucleotidyl transferase
tdTomato : Tandem dimer Tomato
TEL : Telomere maintenance
TID : Two-hyrid interaction with DMC1
T-NOS : Terminateur de la nopaline synthétase
TOP : Topoisomérase
UV : Ultraviolet
V(D)J : Variable (Diversity) Joining
WT : Wild-type
XLF : XRCC4 like factor
XPF : Xeroderma pigmentosum complementing group F
XRCC : X-ray repair cross-complementing
YFP : Yellow fluorescent protein

Notations

Allèle sauvage des gènes : Gene
Allèle mutant des gènes : *gene*
Protéine de mammifères et de plantes : PROTEINE
Protéine de levure : Protéine

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Synthèse bibliographique

Introduction

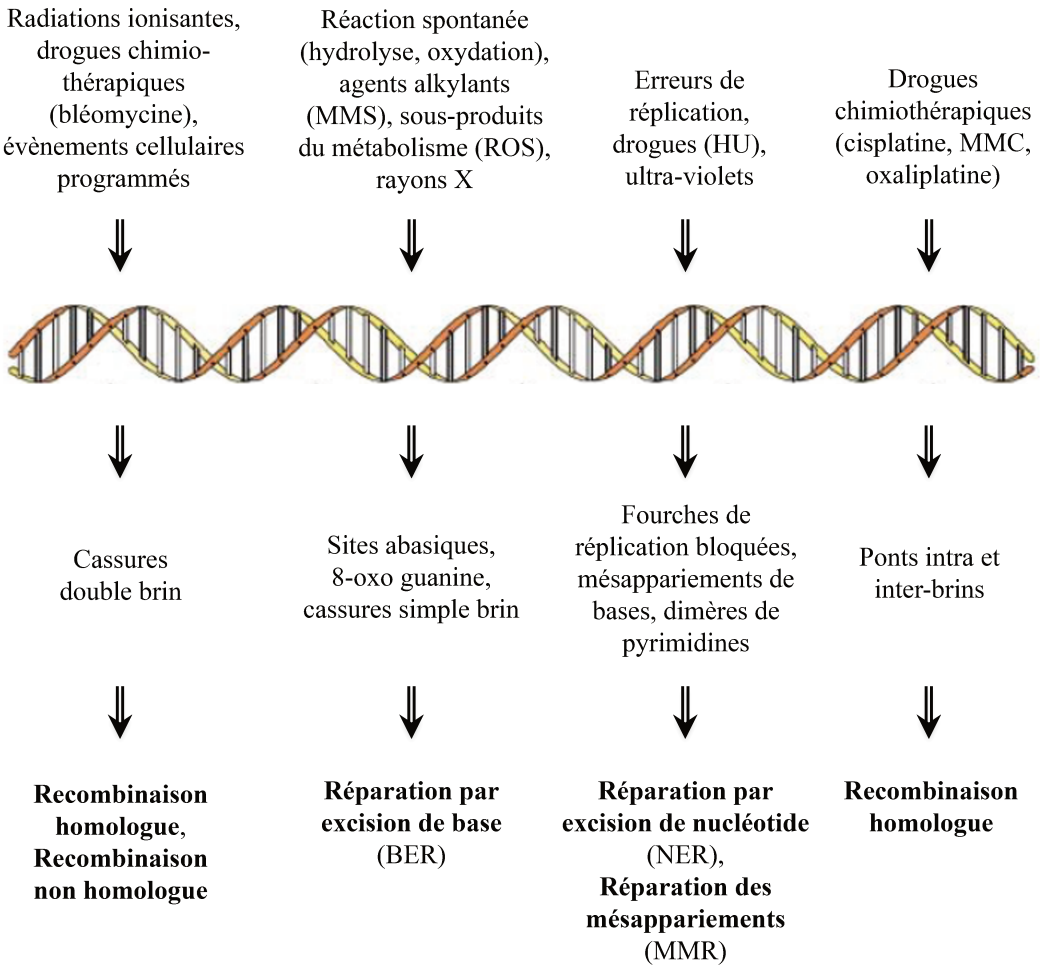


Figure 1 : **Relations entre agents mutagènes, lésions de l'ADN et systèmes de réparation** (d'après Ghosal *et al.*, 2013)

MMS *(Méthyl Méthane Sulfonate)*, ROS *(Reactive Oxygen Species)*, HU *(Hydroxyurea)*, MMC *(Mitomycine C)*

L'ADN est le support de l'information génétique, nécessaire au développement et au fonctionnement de tous les êtres vivants. Le maintien de l'intégrité du génome est fondamental pour assurer la survie de l'organisme, mais également la transmission correcte de son patrimoine génétique à ses descendants. Cependant, les molécules d'ADN sont en permanence endommagées par de nombreux agents d'origine exogène (radiations ionisantes, agents chimiques...) et endogène (produits génotoxiques issus du métabolisme cellulaire notamment). Le nombre de lésions générées chez l'Homme est estimé à plusieurs milliers par cellule et par jour (Lindahl & Barnes, 2000). Celles-ci sont de natures très diverses : altérations des bases ou du squelette désoxyribophosphate (désamination, alkylation, oxydation), ponts intra ou inter-brins, cassures simple ou double brin de la molécule d'ADN. En réponse à ces dommages, un réseau coordonné de cascades de signalisation se met en place à l'échelle cellulaire. Après la détection des lésions de l'ADN, les points de contrôle du cycle cellulaire sont activés afin de retarder la progression du cycle cellulaire et permettre la réparation des dommages *via* le recrutement de la machinerie de réparation de l'ADN. Cet ensemble de processus constitue la réponse aux dommages de l'ADN.

Plusieurs systèmes de réparation de l'ADN prennent en charge les différents types de lésions : la réparation par excision de base (BER), la réparation par excision de nucléotide (NER), la réparation des mésappariements (MMR), la recombinaison homologue et la recombinaison non homologue - ces deux derniers processus étant impliqués dans la réparation des cassures double brin (CDB) (**Figure 1**) (Ciccia & Elledge, 2010 ; Symington & Gautier, 2011 ; Kamileri *et al.*, 2012 ; Dianov & Hübscher, 2013). La recombinaison homologue intervient également dans le redémarrage des fourches de réplication bloquées (pour revues, voir Branzei & Foiani, 2007 ; Petermann & Helleday, 2010). De nombreuses études ont porté sur l'identification et la caractérisation des protéines mises en jeu dans ces voies de réparation, et ont mis en évidence une grande conservation de ces activités au sein

des Procaryotes et des Eucaryotes. Ceci reflète le rôle essentiel des systèmes de réparation dans le maintien de l'intégrité du génome.

Mes travaux de thèse se sont focalisés sur les voies de réparation des cassures double brin de l'ADN, lésions les plus cytotoxiques puisqu'elles fragmentent les chromosomes. Après avoir décrit les origines des CDB de l'ADN, nous présenterons les conséquences possibles d'une absence ou d'une mauvaise réparation de ces cassures. Les principaux mécanismes de réparation des CDB seront ensuite présentés : les voies de recombinaison non homologue, ou voies de jonction des extrémités de la cassure, dans un premier temps, puis les voies de recombinaison homologue, c'est-à-dire impliquant une homologie de séquences pour la réparation.

Chapitre I

Les cassures double brin de l'ADN : causes et conséquences

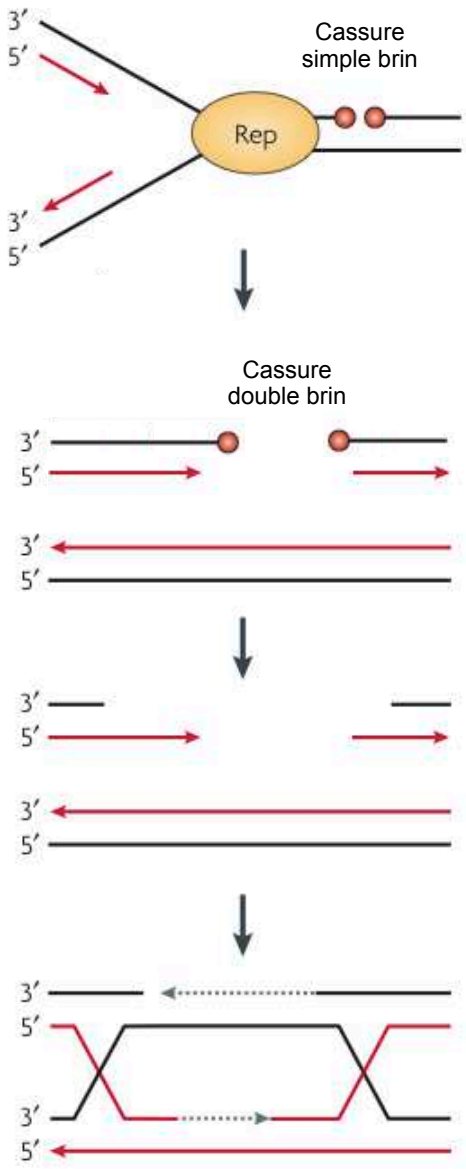


Figure 2 : Conversion d'une cassure simple brin en cassure double brin au cours de la réplication de l'ADN (d'après Caldecott *et al.*, 2008)

Cet exemple présente le cas d'une CSB du brin avancé ; le résultat serait similaire si celle-ci touchait le brin retardé (Rep : fourche de réplication ; lignes rouges : brins nouvellement synthétisés).

I. Origines des cassures double brin de l'ADN

I.1. Les cassures double brin accidentelles

Les cassures double brin (CDB) de l'ADN peuvent être la conséquence de l'action d'agents génotoxiques environnementaux (radiations ionisantes, produits radiomimétiques tels que la bléomycine) ou d'agents issus du métabolisme cellulaire (notamment les espèces réactives de l'oxygène : ion superoxyde, peroxyde d'hydrogène, radical hydroxyle). Des CDB sont également générées au cours des processus cellulaires : tensions dans la molécule d'ADN, accidents de réplication ou de réparation, action de nucléases. De plus, une exposition prolongée à des agents mutagènes ou une déficience des systèmes de réparation aboutit à la persistance d'autres types de lésions de l'ADN, qui peuvent être converties en CDB au cours de la réplication. C'est le cas des dimères de thymine, des ponts inter-brins et des cassures simple brin (CSB) (**Figure 2**). La rencontre d'une CSB par une fourche de réplication peut également générer une CDB à une seule extrémité d'ADN, comme discuté par Helleday *et al.* (2007).

A titre d'exemple, la létalité cellulaire induite par les rayonnements ionisants est liée aux nombreuses lésions que subissent les molécules d'ADN et notamment aux CDB. Le rendement de cassures produites directement par les rayonnements ionisants est estimé à 35 CDB par cellule diploïde humaine (en G1) et par Gray, contre environ 1000 CSB localisées sur les deux brins de la molécule d'ADN (Ward, 1988). Ces rayonnements sont également responsables de l'oxydation de nombreuses bases situées dans les mêmes régions que les CSB, formant ainsi des groupes de lésions (Sutherland *et al.*, 2000 ; Sutherland *et al.*, 2002). Ceux-ci sont plus difficiles à réparer que des lésions simples (Dianov *et al.*, 2001). Certaines lésions peuvent dans un second temps être converties en CDB, à travers l'action des enzymes impliquées dans la réparation par excision de base, ou au niveau des fourches de réplication de l'ADN, quand la machinerie de réplication rencontre ces lésions groupées. Cette capacité des rayonnements

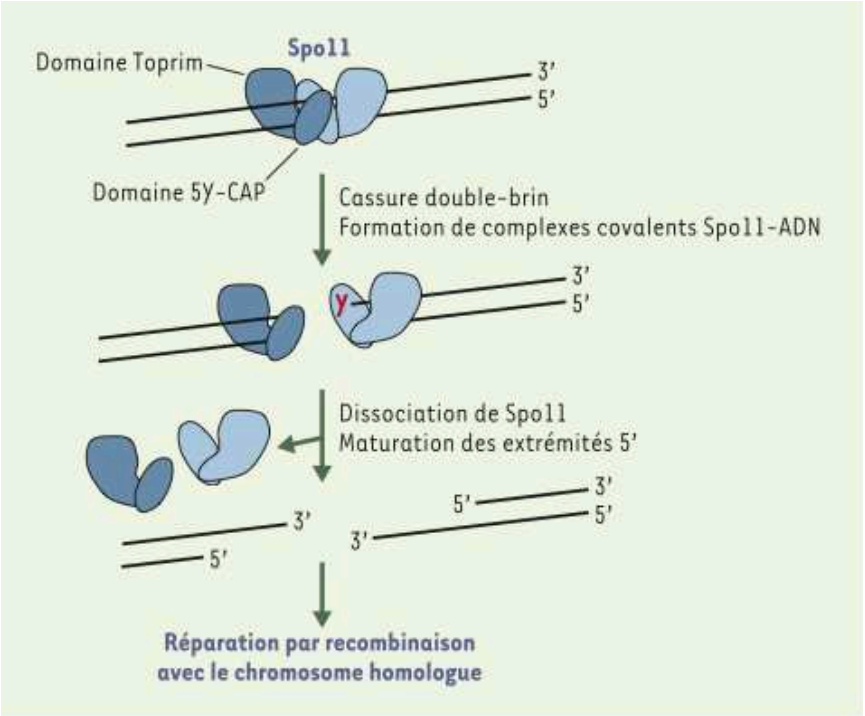


Figure 3 : Déclenchement de la recombinaison méiotique par la formation d'une cassure double brin catalysée par SPO11 (d'après Baudat & De Massy, 2004)

Le domaine carboxy-terminal Toprim (commun aux topoisomérases et aux primases) de chaque monomère de SPO11 interagit avec le domaine 5Y-CAP *(catabolite gene activator protein)* de l'autre monomère. Un dimère se fixe sur l'ADN, introduit une CSB par l'intermédiaire des tyrosines catalytiques (Y) situées dans les domaines 5Y-CAP et forme deux intermédiaires covalents avec les deux extrémités 5' d'ADN. A ce stade, les monomères peuvent rester associés ou se séparer. SPO11 se dissocie ensuite de l'ADN. Les cassures double brin ainsi formées sont ensuite réparées par recombinaison homologue.

ionisants (ainsi que des agents chimiques tels que la bléomycine) à induire des CDB extrêmement délétères est exploitée dans de nombreux traitements anticancéreux pour éliminer les cellules tumorales.

I.2. Les cassures double brin programmées

A ces CDB d'origine accidentelle s'ajoutent des cassures dites programmées. Celles-ci sont générées par des enzymes cellulaires de manière extrêmement contrôlée et sont essentielles dans plusieurs processus du développement ou de la différenciation cellulaire.

I.2.1. Au cours de la méiose : rôle de l'enzyme SPO11

La ségrégation correcte des chromosomes homologues au cours de la première division de méiose ainsi que l'échange de matériel génétique (conversion génique et crossing-over) sont dépendants de la formation de CDB (Cao *et al.*, 1990 ; Page & Hawley, 2003). Ces CDB méiotiques sont générées au stade leptotène de la prophase I par l'enzyme SPO11 (**Figure 3**) (Bergerat *et al.*, 1997 ; Keeney *et al.*, 1997). Cette protéine est extrêmement bien conservée chez les champignons, les animaux et les plantes (pour revue, voir Keeney, 2008). Le rôle fondamental de SPO11 en méiose est révélé par le phénotype méiotique sévère des mutants : d'une fertilité fortement réduite chez *S. pombe* et *A. thaliana* à une stérilité totale chez la souris (pour revue, voir Baudat & de Massy, 2004). L'induction de ces CDB au cours de la méiose est donc essentielle à la production de gamètes viables et à la reproduction.

Des analyses plus récentes ont montré que les CDB méiotiques ne se produisent pas aléatoirement, mais sont localisées préférentiellement dans certaines régions du génome, appelées "points chauds" (Pan *et al.*, 2011 ; Smagulova *et al.*, 2011). Le domaine de liaison à l'ADN de SPO11 ne présente pas ou peu de spécificité de séquence mais l'activité de cette enzyme est dépendante de nombreux facteurs (pour revue, voir Borde & de Massy, 2013). La structure de la chromatine joue également un rôle clé dans la régulation de la formation des

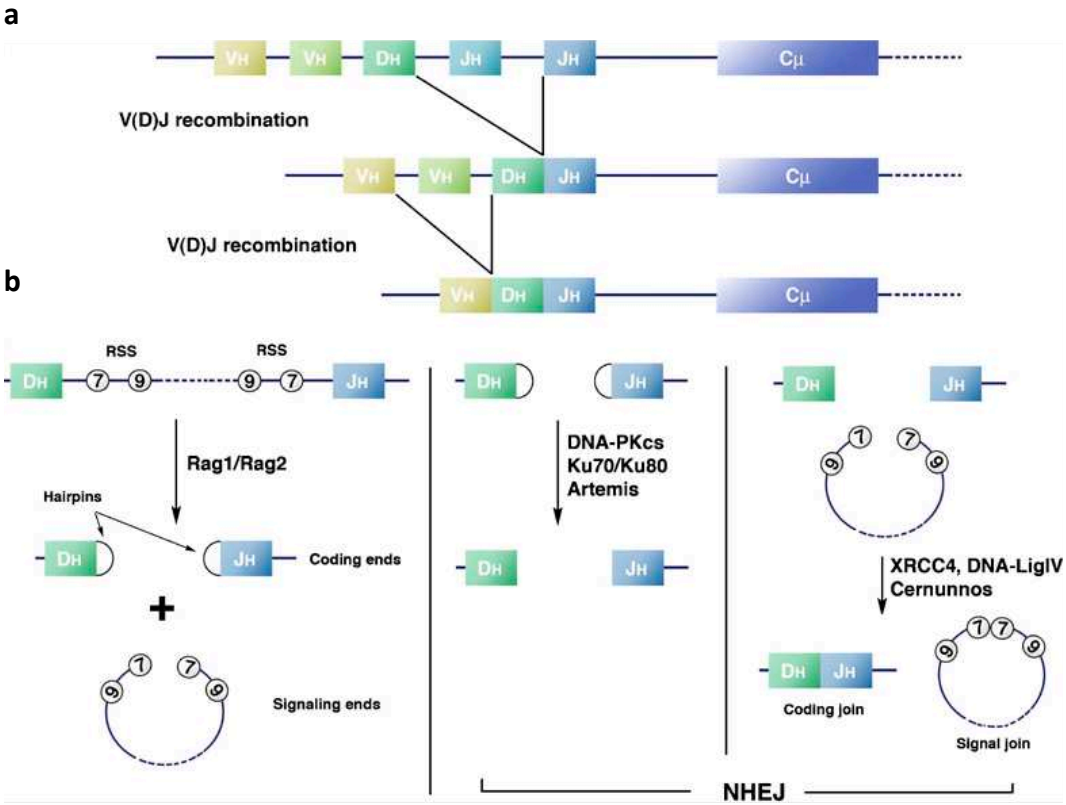


Figure 4: Induction d'une CDB par RAG1 et RAG2 et recombinaison V(D)J (d'aprsè Soulas-Sprauel *et al.*, 2007)

(a) Représentation schématique du locus de la chaîne IgH (Immunoglobuline H) et processus de la recombinaison VDJ.

(b) La recombinaison V(D)J peut être résumée en trois étapes. Le complexe RAG1/RAG2 réalise une coupure à la bordure entre les ségments VH et DH et leurs RSS respectifs, générant ainsi une CDB avec des extrémités en épingle à cheveux (hairpins). Le complexe KU70-KU80 reconnaît la cassure, la DNA-PKcs et Artemis permettent la maturation des extrémités d'ADN, puis le complexe XRCC4-LigaseIV-XLF assure leur jonction. RSS *(recombination signal sequence)* ; NHEJ *(non homologous end joining)* : voie de recombinaison non homologue dépendante de l'hétérodimère KU.

CDB méiotiques en influençant l'activité de SPO11 (aussi bien à l'échelle locale des points chauds qu'à l'échelle chromosomique) (pour revue, voir Yamada & Ohta, 2013). Puisque ces CDB programmées initient la recombinaison méiotique (mécanismes détaillés dans le chapitre III), elles sont à l'origine des crossing-over et des évènements de conversion génique participant à la variabilité génétique des gamètes.

I.2.2. Au cours de la différenciation des lymphocytes B et T : rôle de RAG1 / RAG2

L'induction de CDB dans la région des gènes codant pour les régions variables des immunoglobulines et des récepteurs des lymphocytes constitue un autre exemple de CDB programmées chez les Vertébrés. Elle se produit dans les cellules B et T immatures (futures lymphocytes) et correspond à l'étape initiale de la recombinaison V(D)J. La génération de ces CDB est attribuée à RAG1 et RAG2, deux protéines exprimées uniquement durant la phase de différenciation des lymphocytes B et T (Schatz *et al.*, 1989 ; Oettinger *et al.*, 1990 ; Van Gent *et al.*, 1996). Elles sont capables de se lier spécifiquement aux régions RSS *(recombination signal sequence)* et d'induire une CDB de la double hélice d'ADN entre une région RSS et un segment codant V *(variable)*, D *(diversity)* ou J *(joining)*. Les CDB générées sont prises en charge par la voie de recombinaison non homologue dépendante des protéines KU (mécanismes détaillés dans le chapitre II) et permet la liaison de segments codants non contigus (**Figure 4**).

Ces réarrangements génomiques sont à l'origine du vaste répertoire des immuno-globulines et des récepteurs des lymphocytes B et T, impliqués dans la reconnaissance spécifique d'une grande diversité d'antigènes que peut rencontrer l'individu au cours de sa vie. L'induction de ces CDB (puis leur réparation par recombinaison) est donc essentielle à la réponse immunitaire acquise des Vertébrés (pour revue, voir Nishana & Raghavan, 2012).

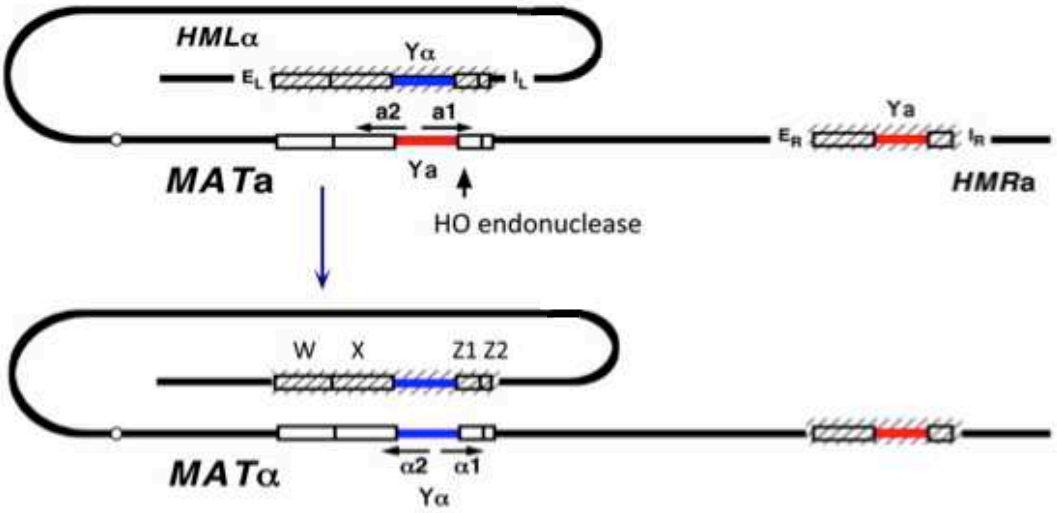


Figure 5 : Changement de type sexuel chez *Saccharomyces cerevisiae* (d'après Haber, 2012)

L'endonucléase HO réalise un cassure double brin entre les régions Y et Z1 au locus MAT. Après maturation des extrémités de la cassure, la région simple brin envahit le locus donneur HML (dans l'exemple présenté ici) et amorce une synthèse d'ADN qui permet de remplacer entièrement la région Y du locus MAT. Zone hachurée : région hétérochromatique.

I.2.3. Au cours de la commutation de type sexuel chez *S. cerevisiae* : rôle de HO

La levure *Saccharomyces cerevisiae* a la particularité de changer de type sexuel au cours de sa croissance végétative à l'état haploïde. La commutation de type sexuel est un mécanisme initié par l'induction d'une CDB par l'endonucléase HO *(homothallic switching endonuclease)* au niveau d'une séquence spécifique (Strathern *et al.*, 1982 ; Kostriken *et al.*, 1983). Cette séquence se situe sur le chromosome III au niveau du locus MAT *(mating-type)*, entre les régions Y et Z1 (**Figure 5**). Bien que cette séquence spécifique soit également présente au niveau des loci hétérochromatiques HML et HMR, aucun clivage n'a lieu du fait de l'inaccessibilité de ces régions à l'enzyme HO (**Figure 5**, régions hachurées). L'un de ces loci sert de matrice à l'évènement de recombinaison homologue de type conversion génique que subi le locus MAT (mécanismes moléculaires détaillés dans le chapitre III) (pour revue, voir Haber, 2012).

La commutation de type sexuel chez la levure est dépendante de l'induction de la CDB programmée par l'endonucléase HO, donc directement liée au profil de transcription du gène codant cette enzyme. L'expression de ce gène est hautement contrôlée et n'est active que dans la cellule mère (à la fin de la phase G1) (Nasmyth, 1993). Ainsi, seule la cellule mère peut subir un changement de type sexuel (MATa vers MATα ou inversement). Les deux cellules de type sexuel différent peuvent alors fusionner, générant ainsi un zygote diploïde a/α pouvant subir la méiose (pour revue, voir Haber, 2012). Ce processus permet à une spore haploïde isolée de devenir rapidement diploïde et de se multiplier par bourgeonnement jusqu'à ce que les conditions environnementales (appauvrissement du milieu) l'amène à sporuler.

Depuis sa découverte, l'endonucléase site-spécifique HO s'est avérée être un outil de choix pour l'étude des mécanismes de recombinaison *in vitro* et *in vivo*.

II. Conséquences de l'absence ou d'une mauvaise réparation des cassures double brin d'ADN

L'absence de réparation d'une CDB de la double hélice d'ADN induit une discontinuité dans la molécule d'ADN pouvant mener à la perte de fragments chromosomiques au cours des divisions cellulaires. Cette perte de matériel génétique, qui concerne parfois un bras entier de chromosome, a des conséquences désastreuses évidentes pour la cellule. Bien que de moindre importance, la présence d'une discontinuité au niveau de la molécule d'ADN affecte également les processus biologiques tels que la réplication et la transcription. Il a été montré par exemple que l'induction d'une seule CDB dans une cellule humaine entraîne l'inhibition de la transcription de la région *via* la dissociation de l'ARN polymérase II de la molécule d'ADN (Pankotai *et al.*, 2012).

La réparation incorrecte des CDB peut engendrer des réarrangements chromosomiques de différentes échelles : de la mutation d'un ou de quelques nucléotides à la duplication ou la translocation de bras chromosomiques. Ces mutations peuvent induire des modifications de l'expression des gènes ayant des conséquences à l'échelle cellulaire, voire de l'organisme. La perte d'un gène suppresseur de tumeur et/ou la surexpression d'un oncogène peuvent être à l'origine d'un processus de tumorigenèse. Les cellules cancéreuses présentent en effet de nombreux réarrangements génomiques (délétions, inversions, duplications et translocations). La progression de la malignité est souvent corrélée à l'augmentation de l'instabilité génomique, résultat de l'escalade des défauts dans les systèmes de réparation de l'ADN et au niveau des points de contrôle du cycle cellulaire (pour revues, voir Hoeijmakers, 2001 ; Jackson & Bartek, 2009 ; Bohgaki *et al.*, 2010).

A titre d'exemple, la translocation réciproque d'un fragment des bras longs des chromosomes 9 et 22 juxtapose l'oncogène Abl *(Abelson leukemia virus tyrosine kinase)* et le gène Bcr *(Breakpoint cluster region)*, générant un oncogène de fusion Bcr-Abl. L'activité

kinase de la protéine de fusion (nettement augmentée par rapport à celle de la protéine ABL) stimule la prolifération incontrôlée des leucocytes, ce qui initie la leucémie myéloïde chronique chez l'Homme. L'accumulation de modifications génétiques et/ou épigénétiques supplémentaires (de manière BCR-ABL dépendante ou indépendante) contribuent à la progression de la maladie dans une phase plus agressive (pour revue, voir Perrotti *et al.*, 2010).

Il semble donc clair que l'absence de réparation d'une CDB ou une réparation incorrecte peut avoir des conséquences dramatiques à l'échelle de la cellule et de l'organisme. Pour lutter contre ces effets néfastes, la cellule dispose d'une machinerie complexe spécialisée dans la réparation des CDB de l'ADN et impliquant l'action coordonnée de nombreuses protéines. Deux grands mécanismes peuvent être distingués selon l'utilisation ou non de séquences homologues pour la réparation : la recombinaison homologue et la recombinaison non homologue.

Chapitre II

Les voies de recombinaison non homologue

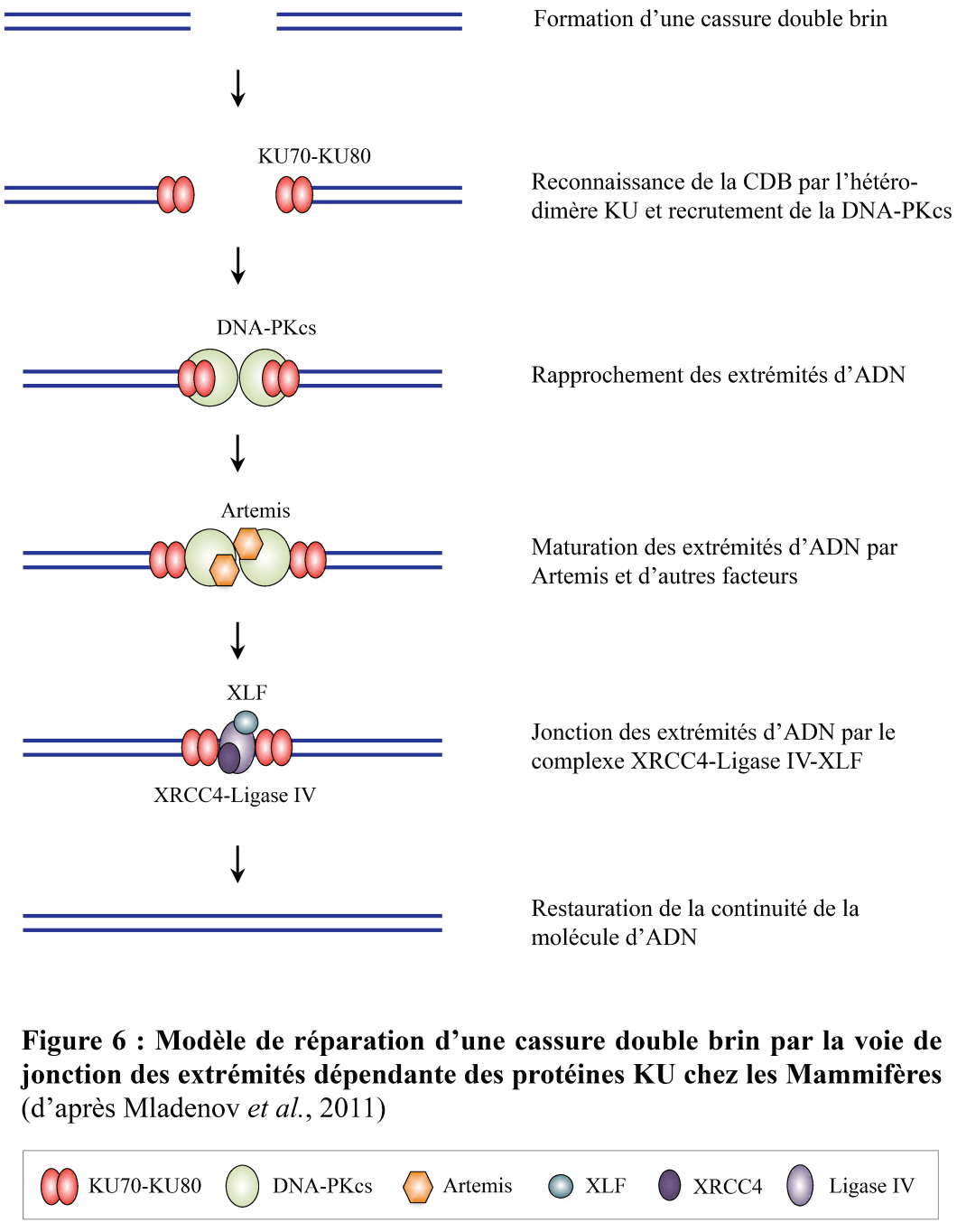


Figure 6 : Modèle de réparation d'une cassure double brin par la voie de jonction des extrémités dépendante des protéines KU chez les Mammifères (d'après Mladenov *et al.*, 2011)

La réparation des CDB par recombinaison non homologue consiste en la jonction des deux extrémités de la cassure. Ce système de réparation est largement majoritaire chez les Eucaryotes pluricellulaires et a été, de ce fait, largement étudié au cours des dernières décennies. La première voie de recombinaison non homologue identifiée est la voie canonique de jonction d'extrémités non homologues (ou C-NHEJ), voie dépendante des protéines KU. Celle-ci existe chez tous les Eucaryotes étudiés et est très bien décrite. Il existe également une ou plusieurs voies indépendantes des protéines KU, qui font actuellement l'objet de nombreuses analyses.

I. Voie de jonction des extrémités dépendante des protéines KU

Cette voie de recombinaison est fonctionnelle à tous les stades du cycle cellulaire mais semble particulièrement active au cours de la phase G1 (Delacôte & Lopez, 2008). Elle est impliquée dans la réparation des CDB mais aussi dans la génération de la diversité des immunoglobulines et des récepteurs des lymphocytes B et T *via* la recombinaison V(D)J (Soulas-Sprauel *et al.*, 2007) (**Figure 4**). L'inactivation d'un des acteurs de cette voie entraîne un phénotype sévère des mutants (d'une hypersensibilité aux radiations ionisantes à la létalité embryonnaire), ce qui met en évidence le rôle essentiel de ce type de réparation (pour revue, voir Brugmans *et al.*, 2007).

Les protéines majeures de cette voie et leurs fonctions sont extrêmement bien conservées chez les Eucaryotes, bien que les homologues de certaines des protéines décrites chez les Mammifères n'ont pas été identifiés chez tous les organismes. Le C-NHEJ, ou voie de jonction des extrémités dépendante des protéines KU, peut être subdivisé en trois étapes principales : la reconnaissance de la CDB, la maturation des extrémités d'ADN puis la jonction de ces extrémités, restaurant l'intégrité de la molécule d'ADN. Chacune de ces étapes implique l'action coordonnée de plusieurs protéines (**Figure 6**).

I.1. Reconnaissance de la cassure double brin

L'hétérodimère KU, composé des sous-unités KU70 et KU80, est capable de détecter et de se lier aux extrémités d'ADN avec une haute affinité et de manière indépendante de la séquence nucléotidique (Mimori & Hardin, 1986 ; Paillard & Strauss, 1991). Les deux sous-unités forment un anneau qui encercle les extrémités d'ADN (Walker *et al.*, 2001 ; Tamura *et al.*, 2002), les protégeant ainsi de la dégradation par des exonucléases. Il a en effet été montré que l'inactivation de KU chez la souris ou la levure provoque une dégradation accélérée des extrémités des CDB (Liang & Jasin, 1996 ; Lee *et al.*, 1998).

L'hétérodimère est également responsable du recrutement de la DNA-PKcs *(DNA-dependent protein kinase catalytic subunit)* au niveau de la CDB (Uematsu *et al.*, 2007) et stimule son activité kinase (Gottlieb & Jackson, 1993). Le complexe multiprotéique ainsi formé stabilise les extrémités d'ADN et assure leur rapprochement (Getts & Stamato, 1994 ; Rathmell & Chu, 1994). Malgré la présence de KU, aucun orthologue de la DNA-PK n'a cependant été identifié chez les plantes et les levures.

I.2. Maturation des extrémités d'ADN

Selon la nature des extrémités (franches ou cohésives, 5' ou 3' sortantes, présence ou absence de modifications...), différents facteurs sont ensuite recrutés. La protéine Artemis est une endonucléase agissant au niveau des extrémités en épingle à cheveux, 3' et 5' sortantes, et 3'-phosphoglycolate (Ma *et al.*, 2002 ; Povirk *et al.*, 2007). Artemis interagit avec la DNA-PKcs qui stimule son activité endonucléasique (Ma *et al.*, 2002). Bien qu'une activité exonucléasique d'Artemis ait été suggérée, elle a récemment été remise en question (Pawelczak & Turchi, 2010). Aucun homologue d'Artemis n'a été identifié chez la plante modèle *Arabidopsis thaliana* à ce jour. La protéine PNKP *(polynucleotide kinase /phosphatase)* catalyse la phosphorylation des extrémités 5'-OH et élimine les groupements

phosphate des extrémités 3' (Chappell *et al.*, 2002). Des études récentes ont révélé que, en plus de son rôle essentiel dans la détection des CDB, KU joue un rôle direct dans la maturation de certaines extrémités modifiées *via* son activité lyase 5'-dRP/AP *(5′-desoxyribose-5-phosphate / apurinic/apyrimidic sites)* (Roberts *et al.*, 2010 ; Strande *et al.*, 2012). Les lacunes nucléotidiques éventuellement générées au cours de la maturation des extrémités sont comblées par les ADN polymérases μ, λ et TdT *(terminal deoxynucleotidyl transferase)* chez les Vertébrés (Capp *et al.*, 2006 ; Andrade *et al.*, 2009). TdT agit spécifiquement au cours de la recombinaison V(D)J, son expression étant restreinte au développement lymphocytaire (Gilfillan *et al.*, 1995). Chez les plantes et les levures, ces lacunes sont prises en charge par les ADN polymérases Pol λ et Pol IV, respectivement (Wilson & Lieber, 1999 ; Roy *et al.*, 2013).

I.3. Jonction des extrémités d'ADN

Une fois que des extrémités compatibles sont générées, le complexe XRCC4-Ligase IV permet leur jonction grâce à la formation d'une liaison covalente entre les deux molécules (Grawunder *et al.*, 1997 ; Grawunder *et al.*, 1998). En effet, XRCC4 interagit physiquement avec la Ligase IV avec une haute affinité (Critchlow *et al.*, 1997 ; West *et al.*, 2000 ; Sibanda *et al.*, 2001 ; Wu *et al.*, 2009), permet la stabilisation de la protéine et stimule son activité (Grawunder *et al.*, 1997). Ce complexe et son activité sont conservés chez les plantes et les levures (complexe Dnl4-Lif1 *(DNA ligase 4 - ligase interfacing factor 1)*).

La protéine XLF (*XRCC4 like factor*, aussi nommé Cernunnos) est également capable d'interagir avec le complexe XRCC4-Ligase IV, mais avec une plus faible affinité (Riballo *et al.*, 2009). Cette interaction stimulerait l'activité du complexe (Ahnesorg *et al.*, 2006 ; Lu *et al.*, 2007). Des études *in vitro* ont suggéré que XLF serait seulement nécessaire à la réparation d'un sous-ensemble de CDB impliquant des mésappariements ou des extrémités non-

cohésives (Tsai *et al.*, 2007). XLF est conservé chez *S. cerevisiae* (Nej1) mais le génome d'*Arabidopsis* ne semble pas posséder d'orthologue de XLF (Kegel *et al.*, 2001).

Le recrutement de XRCC4-Ligase IV et de XLF au niveau du site de la cassure est dépendant de l'hétérodimère KU mais indépendant de la DNA-PK (Mari *et al.*, 2006 ; Yano *et al.*, 2008). Ceci indique que KU est non seulement impliqué dans la détection de la cassure et la maturation des extrémités d'ADN, mais également indirectement dans la dernière étape du C-NHEJ (pour revue, voir Radhakrishnan *et al.*, 2014).

I.4. Fidélité de la réparation

Il a été établi que la voie de jonction des extrémités dépendante de KU favorise la stabilité génomique en limitant les translocations chromosomiques (Simsek & Jasin, 2010). En effet, au cours de la réparation d'une CDB par C-NHEJ, les deux extrémités d'ADN sont très rapidement prises en charge et maintenues ensemble par le complexe protéique KU-DNA-PK. Ainsi, dans la majorité des cas, les deux extrémités d'ADN initialement contiguës sont liées entre elles (pour revues, voir Iliakis *et al.*, 2004 ; Lieber, 2010 ; Gostissa *et al.*, 2011). En revanche, le C-NHEJ est traditionnellement considéré comme un mécanisme générant des erreurs puisque les étapes de maturation des extrémités modifient la séquence nucléotidique au niveau de la CDB. Cependant, de nombreuses études suggèrent que les protéines KU protègent les extrémités d'ADN contre une dégradation étendue, limitant ainsi les effets néfastes potentiels des mutations de type délétion (Guirouilh-Barbat *et al.*, 2004 ; Schulte-Uentrop *et al.*, 2008). La voie du C-NHEJ est donc un mécanisme de réparation générant relativement peu d'erreurs (à l'échelle nucléotidique) et évitant les mutations de plus grande échelle.

II. Voie(s) de jonction des extrémités indépendante(s) des protéines KU

La détection de produits de réparation de type jonction d'extrémités dans des cellules déficientes pour *ku80* a permis de mettre en évidence l'existence d'une ou de plusieurs voies de réparation indépendante(s) de KU (Liang *et al.*, 1996 ; Kabotyanski *et al.*, 1998). L'analyse de ces produits, présentant majoritairement des délétions ou insertions de quelques nucléotides, implique des remaniements au niveau des extrémités de la CDB et suggère une réparation par microhomologies (c'est-à-dire *via* l'appariement de quelques bases situées de part et d'autre de la CDB) (Kramer *et al.*, 1994 ; Moore & Haber, 1996 ; Feldmann *et al.*, 2000). Cette (ces) voie(s) semble(nt) particulièrement active(s) en absence de la voie dépendante de KU (Soulas-Sprauel *et al.*, 2007 ; Yan *et al.*, 2007). Cependant, le nombre de voies indépendantes des protéines KU, ainsi que les protéines impliquées sont encore mal définies. De multiples noms ont été utilisés dans la littérature : alternative NHEJ (aNHEJ ou alt-NHEJ), back-up NHEJ (bNHEJ) et microhomology-mediated end-joining (MMEJ) (Ma *et al.*, 2003 ; Corneo *et al.*, 2007 ; Iliakis, 2009). Nous regrouperons ici ces voies sous le terme "Voies de jonction des extrémités indépendantes des protéines KU".

II.1. Les acteurs des voies de jonction des extrémités indépendantes des protéines KU

Plusieurs études ont d'abord suggéré les rôles de la protéine PARP-1 *(Poly(ADP-Ribose) Polymerase 1)*, de la Ligase III et de XRCC1 *(X-ray Repair Cross-Complementing protein 1)* dans les voies indépendantes des protéines KU (pour revue, voir Mladenov & Iliakis, 2011). Ces trois protéines sont par ailleurs impliquées dans la réparation des CSB et dans le système de réparation par excision de base (pour revue, voir Caldecott, 2003). PARP-1 est capable de se fixer au CSB et aux CDB, mais se lie aux CDB avec une plus haute affinité (Benjamin & Gill, 1980). L'inhibition de PARP-1 diminue l'efficacité de réparation des CDB mais dans une moindre mesure par rapport à l'inhibition de PARP-1 et DNA-PKcs

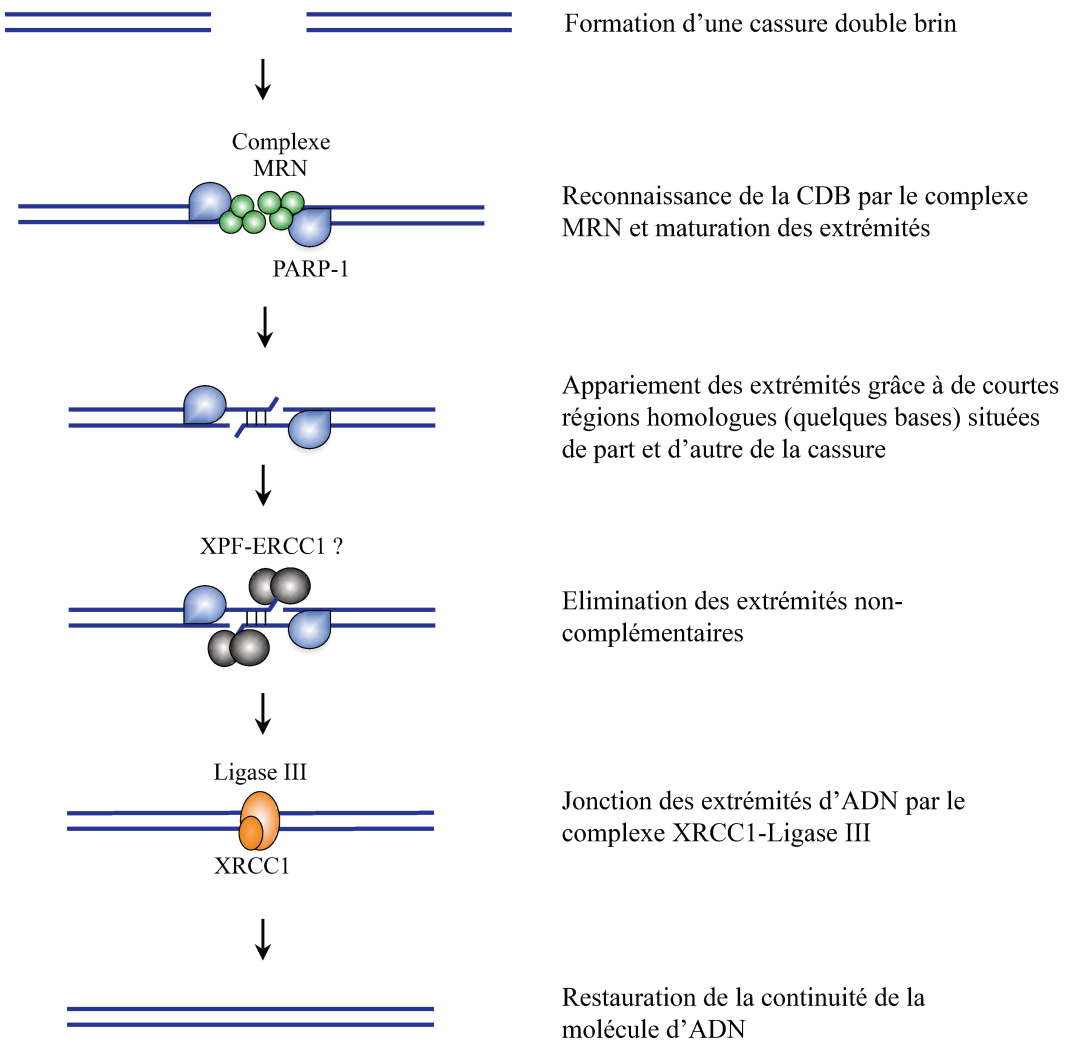


Figure 7 : Modèle de réparation d'une cassure double brin par la voie de jonction des extrémités indépendante des protéines KU chez les Mammifères (d'après Mladenov *et al.*, 2011)

Les principaux acteurs impliqués dans les voies de jonction des extrémités indépendantes de KU sont présentés ici. Il est cependant possible qu'ils interviennent dans des voies parallèles.

(Boulton *et al.*, 1999 ; Veuger *et al.*, 2003 ; Veuger *et al.*, 2004). Ces résultats indiquent que PARP-1 agit dans une voie alternative à la voie C-NHEJ. Plus précisément, PARP-1 serait impliqué dans les premières étapes de la voie (reconnaissance de la CDB et/ou maintien des extrémités) mais son rôle exact reste à identifier (**Figure 7**) (pour revue, voir Ko & Ren, 2012). Le rôle de l'ADN Ligase III dans les voies indépendantes de KU a été mis en évidence *in vitro* et *in vivo* (Wang *et al.*, 2005). La Ligase III interagit physiquement avec XRCC1 et cette interaction stimule l'activité de ligature des brins d'ADN de la ligase (Caldecott *et al.*, 1994). Aubebert *et al.* ont montré que les protéines humaines PARP-1, XRCC1 et la Ligase III sont suffisantes à la réparation d'une CDB par jonction des extrémités *in vitro* (Audebert *et al.*, 2004). Cependant, le rôle de la Ligase III et de XRCC1 dans ces voies indépendantes de KU chez les Mammifères est actuellement controversé (Gao *et al.*, 2011 ; Simsek *et al.*, 2011 ; Boboila *et al.*, 2012 ; Han *et al.*, 2012). D'autres analyses sont donc nécessaires pour comprendre ces apparentes contradictions.

Plusieurs études ont révélé l'implication du complexe MRX (composé des protéines Mre11, Rad50 et Xrs2) dans des voies indépendantes de KU chez la levure (Moore & Haber, 1996 ; Paull & Gellert, 1998 ; Ma *et al.*, 2003). Ce complexe, et plus particulièrement Mre11, favoriserait l'initiation de la réparation en stimulant la résection 5'-3' des extrémités de la CDB - étape nécessaire à la recherche de microhomologies dans les régions bordant la cassure. Un rôle similaire du complexe MRN (MRE11, RAD50, NBS1) a été observé chez les Mammifères (Deriano *et al.*, 2009 ; Rass *et al.*, 2009 ; Rahal *et al.*, 2010).

Un rôle du complexe Rad1-Rad10 de levure dans le clivage des séquences non homologues de part et d'autre des bases appariées a été proposé, mais ce complexe ne serait pas essentiel (Ma *et al.*, 2003). Un test de recircularisation de plasmides chez les Mammifères a également suggéré que XPF-ERCC1, l'homologue de Rad1-Rad10, favoriserait les mécanismes de jonction des extrémités indépendants des protéines KU (Ahmad *et al.*, 2008).

D'autres analyses sont cependant requises, chez les Mammifères comme chez la levure, pour confirmer et préciser le rôle de ces différentes protéines et définir les différentes voies de réparation.

Chez les plantes, des orthologues de MRE11, RAD50, NBS1, XRCC1, XPF et PARP-1 ont été identifiés (Babiychuk *et al.*, 1998 ; Fidantsef *et al.*, 2000 ; Gallego *et al.*, 2001 ; Bundock & Hooykaas, 2002 ; Waterworth *et al.*, 2007 ; Uchiyama *et al.*, 2008). AtMRE11 a été impliqué dans la jonction d'extrémités impliquant des courtes homologies de séquences puisque le nombre de produits de fusion chromosomiques présentant des microhomologies est nettement réduit dans le mutant *mre11* (Heacock *et al.*, 2004). L'utilisation de simple et double mutants chez *Arabidopsis* a révélé que la réparation des CDB radio-induites dans un mutant *ku80* est dépendant de XRCC1 (Charbonnel *et al.*, 2010) et XPF (Charbonnel *et al.*, 2011). Ces protéines sont donc impliquées dans des voies de réparation indépendantes de KU. L'analyse des relations d'épistasie entre ces trois protéines a permis de démontrer que chacune d'elles agit dans une voie indépendante, mettant en évidence pour la première fois l'existence d'au moins deux voies de jonction des extrémités indépendantes des protéines KU chez les plantes (Charbonnel *et al.*, 2011).

II.2. Fidélité de la réparation

Il a été établi que la probabilité de lier deux extrémités d'ADN initialement non contigües au cours de la réparation d'une CDB est nettement augmentée en absence des protéines KU. Les voies de recombinaison non homologue indépendantes de KU sont en effet impliquées dans l'augmentation de l'instabilité génomique (en favorisant notamment les translocations) et donc dans les processus de tumorigenèse (Weinstock *et al.*, 2007). Ceci serait lié à la cinétique de réparation : les voies indépendantes de KU sont plus lentes et moins

efficaces que la voie dépendante de KU, et génèrent donc plus d'erreurs (pour revue, voir Schipler & Iliakis, 2013).

A l'échelle nucléotidique, les voies indépendantes de KU sont à l'origine de mutations de quelques nucléotides à quelques dizaines de nucléotides, principalement des petites délétions ou insertions. Les voies de jonction d'extrémités indépendantes de KU sont donc considérées comme des voies de réparation très mutagènes (pour revues, voir Iliakis *et al.*, 2007 ; Lieber, 2010).

Chapitre III

Les voies de recombinaison homologue

Le terme "recombinaison homologue" au sens large regroupe toutes les voies de réparation des CDB impliquant une homologie de séquence pour la réparation de la molécule endommagée. D'abord, la voie d'hybridation d'ADN simple brin (SSA, *single strand annealing*) permet la réparation des CDB situées entre deux régions homologues. C'est une voie mutagène puisqu'elle aboutit à la délétion de la région située entre les répétitions et d'une des répétitions. En revanche, les voies de recombinaison homologue *sensu stricto* réparent la CDB grâce à la copie d'une séquence homologue non endommagée, située généralement sur la chromatide sœur ou le chromosome homologue. Ces voies de réparations sont dites fidèles puisqu'elles préservent l'intégrité de l'information génétique. Elles sont actives dans les cellules somatiques (principalement au cours des phases S et G2 où la chromatide sœur est présente) mais aussi dans les cellules méiotiques.

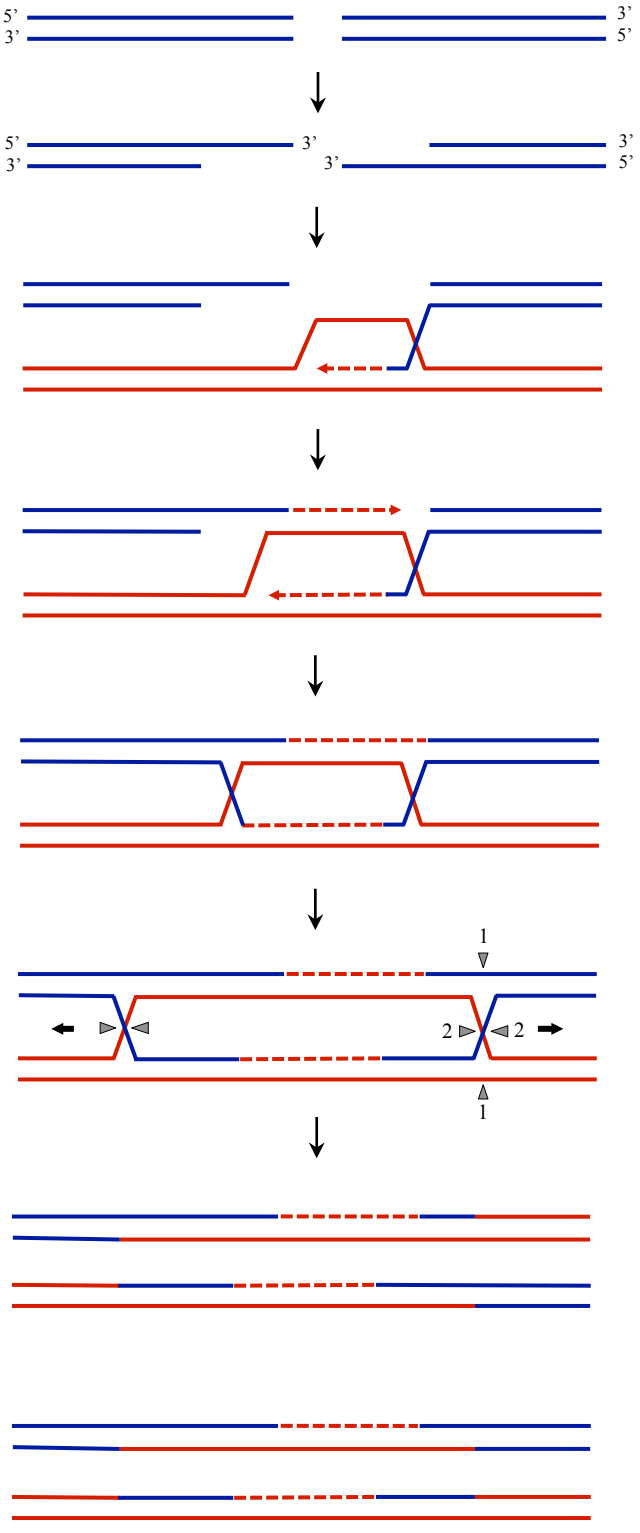
Depuis la proposition du modèle moléculaire de la recombinaison homologue établi par Robin Holliday en 1964, de nouvelles données expérimentales ont permis d'améliorer ce modèle en y apportant des modifications. Ces études ont révélé l'existence de plusieurs voies de recombinaison homologue, plus ou moins indépendantes, impliquant les activités de nombreuses protéines.

I. Mécanismes moléculaires des voies de recombinaison homologue

I.1. Double Strand Break Repair (DSBR)

Le modèle DSBR a été proposé par Jack Szostak *et al.* (1983). Celui-ci est basé sur des études de transformation et de ciblage de gènes chez la levure (Orr-Weaver & Szostak, 1983) et fournit des explications à de nouvelles observations non prises en compte par le modèle de Holliday (Haber *et al.*, 2004).

Les principales étapes de ce modèle sont présentées **figure 8**. Suite à une CDB de l'ADN, les deux extrémités subissent une résection 5'-3' générant des extrémités d'ADN



Formation d’une cassure double brin

Résection 5’- 3’ des extrémités d’ADN

Invasion d’une séquence homologue
Formation de la boucle D

Synthèse d’ADN
Déplacement de la boucle D

Capture de la seconde extrémité
Formation de la double jonction de Holliday

Migration puis résolution des jonctions

Résolution selon (1) génère un échange des régions d’ADN flanquantes (**crossing-over**)

ou

Résolution selon (2) génère un produit de **conversion génique** (non crossing-over)

Figure 8 : Voie DSBR (Double Strand Break Repair)
(d’après Symington, 2002)

simple brin (ADNsb) 3' sortantes. Ces extrémités recherchent une région homologue dans une double hélice d'ADN, généralement la chromatide sœur ou le chromosome homologue. Quand une région homologue est identifiée, l'extrémité d'ADNsb l'envahit par appariement avec le brin complémentaire, formant ainsi une structure appelée boucle de déplacement, ou boucle D. La région d'ADN hétérologue est étendue par une synthèse d'ADN utilisant comme amorce, l'extrémité 3' du brin envahisseur et comme matrice, la copie homologue non endommagée. La poursuite de la polymérisation d'ADN conduit au déplacement de la boucle D qui pourra s'apparier avec la seconde extrémité de la cassure et servir de matrice à son extension. L'intermédiaire ainsi formé est nommé double jonction de Holliday. Les deux simples jonctions peuvent migrer, généralement en sens opposé, conduisant à l'extension du duplexe d'ADN hétérologue (étape de migration des branches). La résolution de cet intermédiaire de recombinaison peut aboutir à différents types de produits selon la localisation des clivages au niveau des deux jonctions (triangles gris, **Figure 8**). Les crossing-over (CO) génèrent un échange réciproque de matériel génétique entre les deux molécules d'ADN homologues. En revanche, les produits de non crossing-over (NCO) résultent d'un transfert non réciproque d'information génétique entre les deux molécules, c'est la conversion génique.

Selon ce modèle, la résolution aléatoire des deux jonctions de Holliday devrait produire un nombre équivalent de produits de CO et de produits de NCO. Or, des études portant sur la commutation de type sexuel chez *S. cerevisiae* (Nasmyth, 1982 ; McGill *et al.*, 1989) ou la recombinaison des éléments transposables chez la drosophile (Gloor *et al.*, 1991) ont révélé que les évènements de conversion génique sont plus nombreux que les CO. Ces travaux ont mené à la proposition d'un nouveau modèle dans lequel la réparation des CDB par recombinaison homologue ne génère que des évènements de conversion génique : il s'agit de la voie SDSA (Synthesis-Dependent Strand-Annealing).

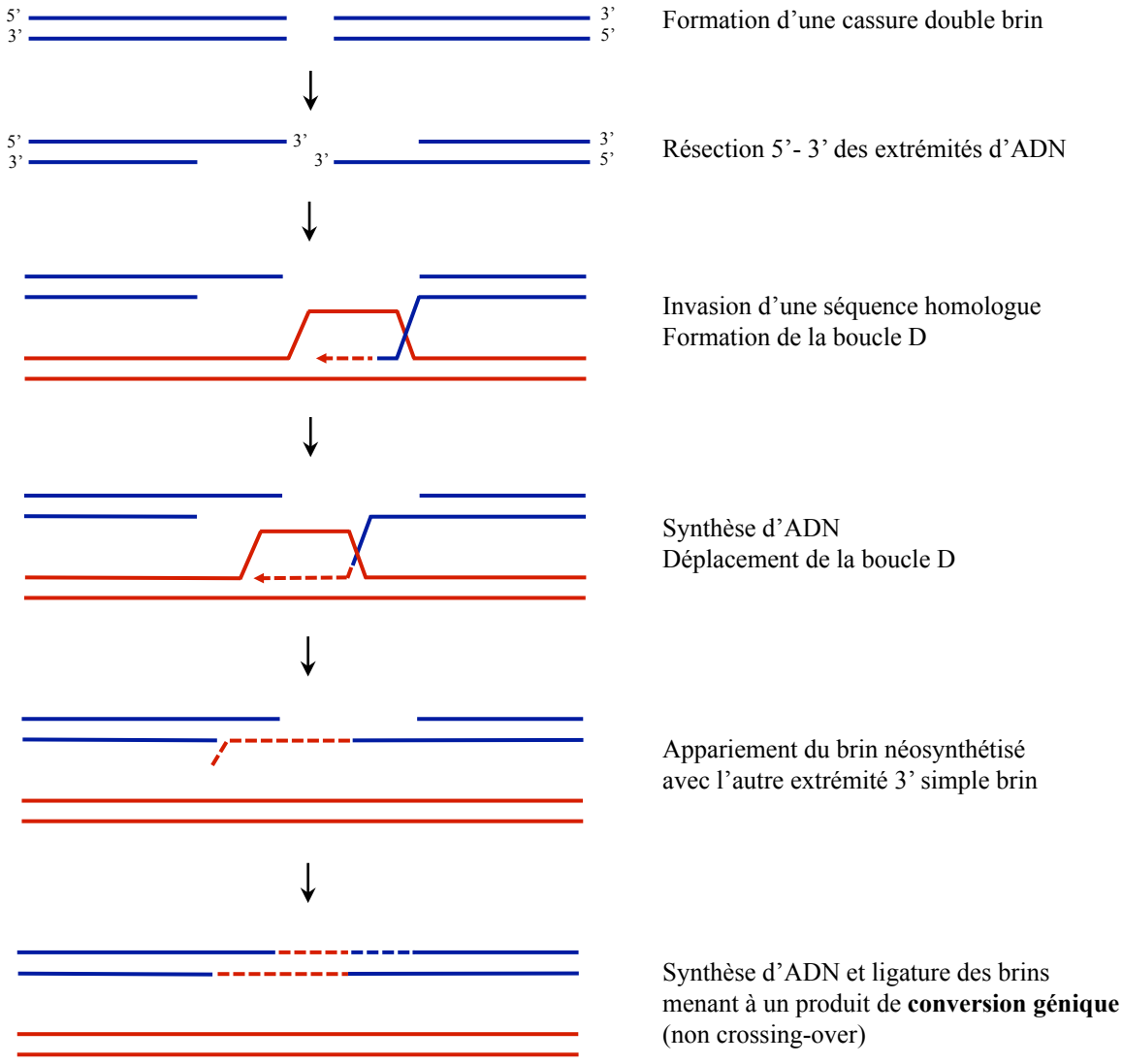


Figure 9 : Voie SDSA (Synthesis-Dependent Strand-Annealing)
(d’après Symington, 2002)

I.2. Synthesis-Dependent Strand-Annealing (SDSA)

Le modèle SDSA, proposé dans les années 90 (Nassif *et al.*, 1994 ; Ferguson & Holloman, 1996), est présenté **figure 9**. Les premières étapes de la voie SDSA sont communes à celles de la voie DSBR : résection 5'-3' des extrémités de la CDB, recherche d'homologie et invasion d'une séquence homologue non endommagée par l'une des extrémités d'ADNsb 3' sortantes. Ceci aboutit à la formation de la boucle D. La synthèse d'ADN initiée à partir de l'extrémité 3' du brin envahisseur provoque le déplacement de la boucle D le long de la matrice d'ADN. Le brin nouvellement synthétisé est ensuite déplacé et peut s'hybrider par complémentarité de bases avec l'autre extrémité d'ADNsb 3' sortante de la chromatide endommagée. La synthèse d'ADN est alors amorcée à partir de l'extrémité 3' du brin non envahisseur et permet de combler les lacunes. La ligature des brins restaure la continuité de la molécule d'ADN.

Une différence essentielle entre ce mécanisme et le modèle de Szostak est qu'il génère exclusivement des produits de NCO (conversion génique), puisqu'il n'implique ni la formation ni la résolution d'une double jonction de Holliday. De plus, l'ensemble de l'ADN nouvellement synthétisé se retrouve finalement dans la molécule "destinataire", c'est-à-dire dans la chromatide initialement endommagée (lignes pointillées, **Figure 9**). En accord avec ces prédictions, les séquences "donneuses" sont généralement non modifiées au cours d'évènements de conversion génique induits par une CDB (Pâques *et al.*, 1998).

Dans les cellules somatiques, la réparation des CDB est assurée principalement par la voie SDSA, prévenant ainsi les potentiels effets négatifs des CO tels que les réarrangements chromosomiques issus d'évènements de recombinaison entre séquences homologues non-alléliques. Les travaux de Allers et Lichten (2001) ont montré que les produits de NCO méiotiques sont principalement générés par SDSA, révélant le rôle clé de cette voie (pour revue, voir Andersen & Sekelsky, 2010).

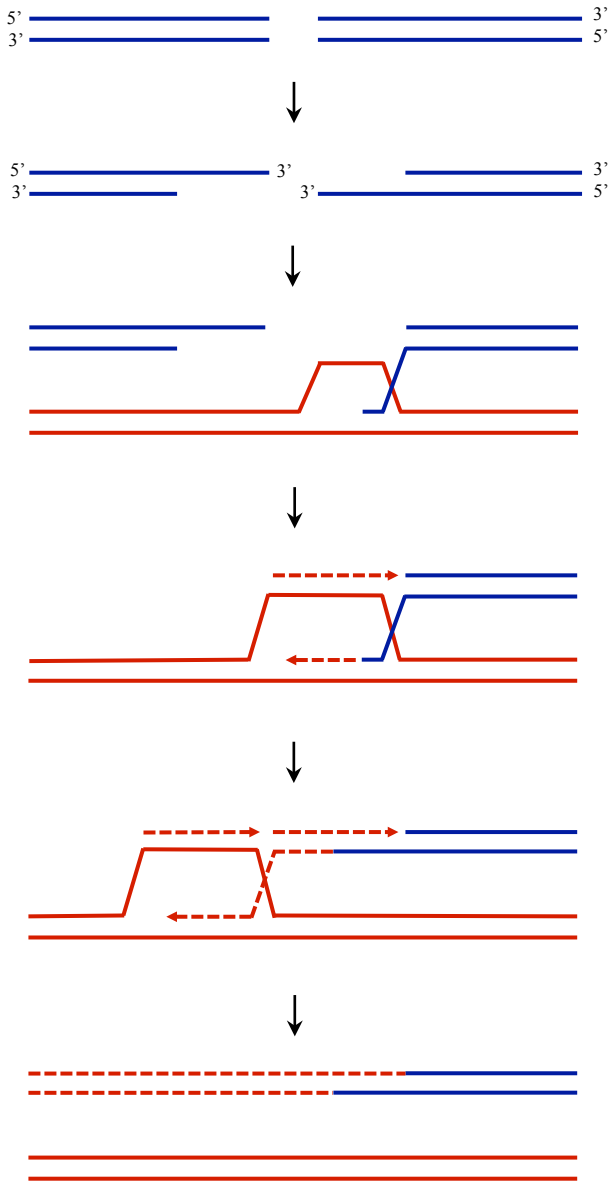


Figure 10 : Voie BIR (Break-Induced Replication)
(d’après Malkova & Ira, 2013)

Alors que les évènements de conversion génique générés par les voies SDSA et DSBR ne concernent que des régions de quelques kilobases, des évènements de plus grande échelle (d'une centaine de kilobases à un bras de chromosome entier) ont été décrits (Esposito, 1978 ; Voelkel-Meiman & Roeder, 1990 ; Malkova *et al.*, 1996). Ces observations ont servi de base à l'élaboration de nouveaux modèles nommés BIR *(Break-Induced Replication)*.

I.3. Break-Induced Replication (BIR)

Inspirés des mécanismes de réplication dépendante de la recombinaison d'*E.coli* et du phage T4, plusieurs modèles de réplication d'ADN induite par une CDB ont été envisagés (Malkova *et al.*, 1996 ; Morrow *et al.*, 1997). Des travaux récents menés chez la levure ont démontré que la voie BIR aboutit à l'héritage conservatif du matériel génétique néosynthétisé, en accord avec le modèle de synthèse d'ADN conservative (Donnianni & Symington, 2013 ; Saini *et al.*, 2013). Comme dans les voies DSBR et SDSA, la voie BIR implique la résection 5'-3' des extrémités de la cassure suivie de la recherche puis de l'invasion d'une séquence homologue (**Figure 10**). La synthèse d'ADN initiée à partir de l'extrémité 3' du brin envahisseur induit la migration de la boucle D. Le mouvement de la jonction non résolue provoque le déplacement du brin nouvellement synthétisé. Ce processus aboutit à la formation d'une molécule d'ADN dont les deux brins sont néosynthétisés, tandis que l'ADN matrice conserve ses deux brins d'origine (Smith *et al.*, 2007). Le BIR peut ainsi être à l'origine de la réplication de grandes régions chromosomiques. La recombinaison de type BIR prendrait en charge les CDB dont une seule extrémité est disponible et serait notamment impliqué dans la réparation des fourches de réplication bloquées et l'allongement des télomères en absence de télomérase (pour revue, voir Malkova & Ira, 2013).

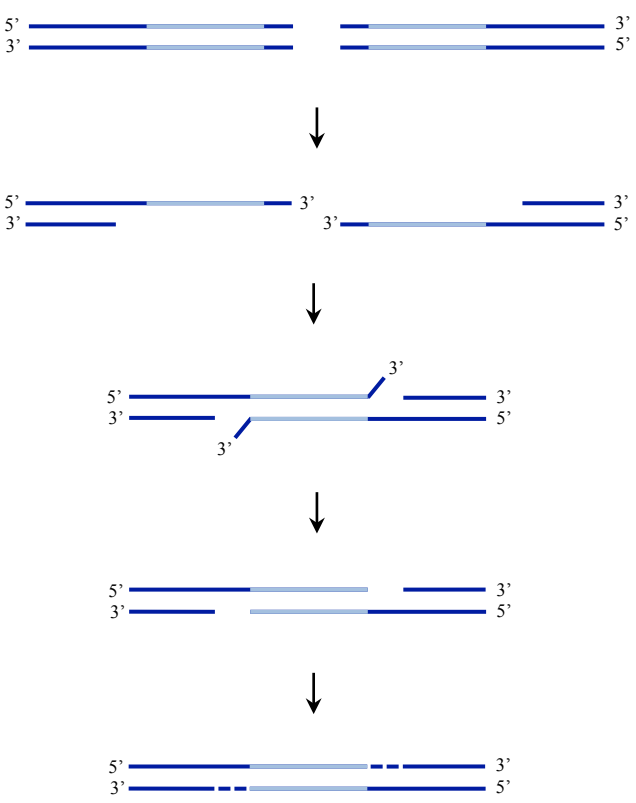


Figure 11 : Voie SSA (Single Strand Annealing)
(d’après Symington, 2002)

I.4. Single-Strand Annealing (SSA)

Une autre voie impliquant une homologie de séquence pour la réparation d'une CDB est la voie d'hybridation simple brin (SSA). Le modèle SSA, proposé initialement par Lin *et al.* (1984), décrit la recombinaison entre deux séquences répétées dans la même orientation situées sur la même molécule d'ADN (SSA intramoléculaire) ou sur deux molécules différentes (SSA intermoléculaire) (Lin *et al.*, 1984). Les principales étapes de la voie SSA intramoléculaire sont présentées dans la **figure 11**. Le processus est initié par la résection 5'-3' des extrémités d'ADN générant des queues simple brin 3' sortantes. Quand la résection est suffisante, les séquences complémentaires situées de part et d'autre de la cassure s'apparient. Les extrémités d'ADNsb non complémentaires sont ensuite clivées et éliminées. La synthèse d'ADN permet de combler les lacunes potentielles avant la ligature des brins. Ce processus aboutit à la délétion du matériel génétique situé entre les séquences répétées ainsi que d'une des répétitions. Contrairement aux voies conservatives précédemment décrites, le SSA est donc un système de réparation mutagène. Il peut également être à l'origine d'importants réarrangements chromosomiques dans le cas où les séquences répétées se situent sur deux chromosomes différents (Richardson & Jasin, 2000).

Des évènements de SSA ont été détectés chez les levures, les animaux et les plantes ; ce mode de réparation des CDB serait particulièrement important dans les génomes présentant de nombreuses séquences répétées (Lin *et al.*, 1984 ; Maryon & Carroll, 1991 ; Fishman-Lobell *et al.*, 1992). L'efficacité du SSA est largement influencée par la longueur des régions répétées, leur degrés d'identité de séquence (Sugawara & Haber, 1992 ; Sugawara *et al.*, 1997) et par la distance séparant les deux répétitions (Fishman-Lobell *et al.*, 1992). A titre d'exemple, il a été montré chez la levure que l'efficacité du SSA est de 100% quand les régions homologues ont une taille supérieure à 400 paires de bases mais seulement de 5% quand celle-ci est de 60 pb (Sugawara & Haber, 1992).

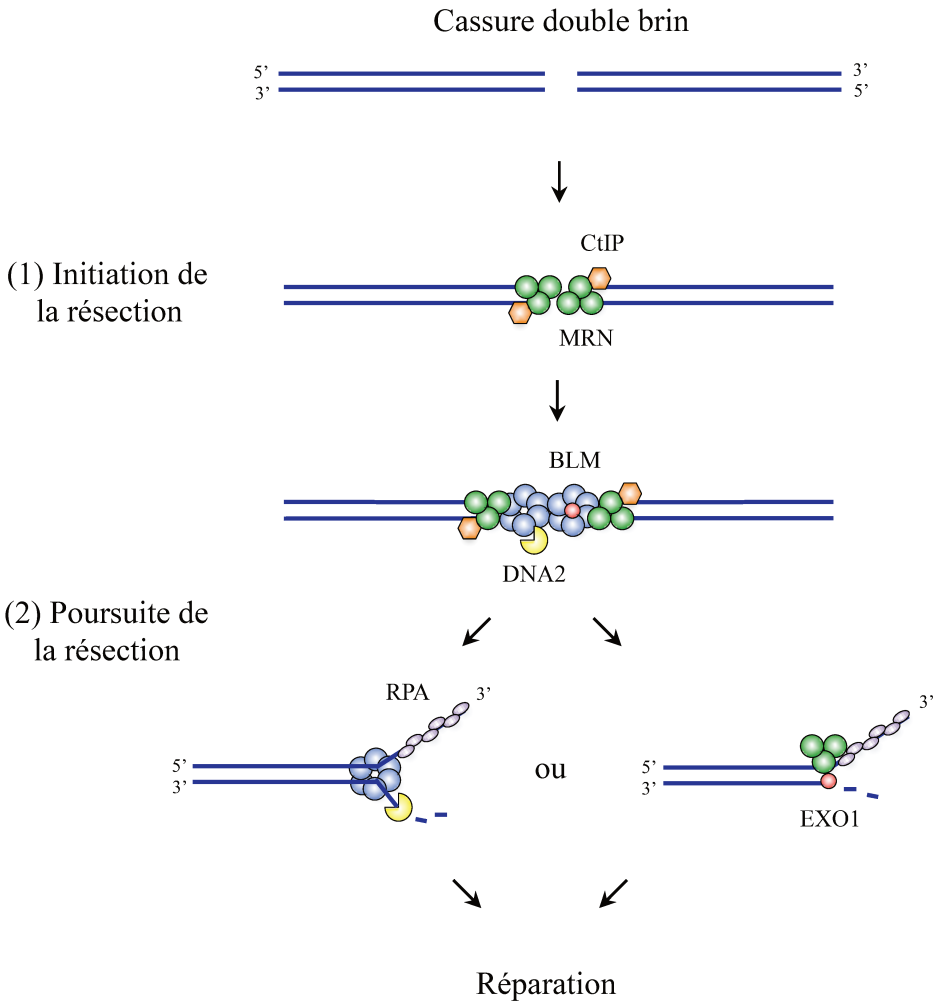


Figure 12 : Mécanisme de la résection des extrémités d'une cassure double brin chez les Mammifères (d'après Nimonkar *et al.*, 2011)

(1) Initiation de la résection. La cassure double brin est reconnue par le complexe MRN qui recrute ensuite CtIP. Le complexe ainsi formé initie la résection sur quelques centaines de nucléotides.

(2) Poursuite de la résection. MRN recrute ensuite BLM-DNA2 ou EXO1-BLM. La résection se poursuit sur plusieurs kilobases grâce à l'activité exonucléasique de DNA2 ou EXO1.

II. Les acteurs de la recombinaison homologue

Les voies de recombinaison homologue (RH) font intervenir un grand nombre de protéines dont les fonctions ont été largement étudiées ces dernières décennies. Pour les Eucaryotes, la majorité de ces protéines ont été identifiées grâce à des expériences de sensibilité aux radiations ionisantes chez *Saccharomyces cerevisiae* ; il s'agit de Rad50, Rad51, Rad52, Rad54, Rdh54 (Tid1), Rad55, Rad57, Rad59, Mre11 (Rad58) et Xrs2. Ces protéines appartiennent au groupe épistatique de Rad52 et sont hautement conservées chez les Eucaryotes (pour revue, voir Krogh & Symington, 2004).

Les principaux acteurs de la recombinaison homologue seront traités chronologiquement, en fonction de l'étape dans laquelle ils interviennent : (1) la reconnaissance et la maturation des extrémités de la CDB, (2) la recherche d'homologie et l'invasion de brin, (3) l'élimination des extrémités non complémentaires, puis (4) la résolution des intermédiaires de recombinaison.

II.1. Les protéines de la reconnaissance et de la maturation des extrémités de la CDB

La détection de la CDB est assurée par le complexe MRN (MRE11-RAD50-NBS1) chez les animaux, ou le complexe MRX (Mre11-Rad50-Xrs2) chez les levures (Lisby *et al.*, 2004 ; Lisby & Rothstein, 2009). Celui-ci se lie rapidement aux extrémités de la CDB et permet dans un premier temps de les maintenir ensemble (De Jager *et al.*, 2001) (**Figure 12**). Il joue ensuite un rôle fondamental dans la signalisation de la cassure en recrutant notamment la protéine ATM *(Ataxia telangiectasia mutated)* (pour revue, voir Lamarche *et al.*, 2010). De plus, le complexe MRN (MRX) participe à l'étape d'initiation de la résection des extrémités de la cassure. Bien que MRE11 possède des activités endonucléase et exonucléase 3'-5' (Paull & Gellert, 1998 ; Trujillo *et al.*, 1998 ; Trujillo & Sung, 2001), aucune activité exonucléase 5'-3' - sens de la résection des extrémités de la cassure - n'a été démontrée. Le complexe MRN

contribuerait à la résection en recrutant ou en facilitant l'activité d'autres facteurs de la RH (pour revue, voir Lamarche *et al.*, 2010). Parmi eux, l'endonucléase CtIP *(CtBP-interacting protein)* (Sae2 chez la levure) initie la résection 5'-3' des extrémités grâce à une interaction physique et fonctionnelle avec le complexe MRN (Sartori *et al.*, 2007). CtIP (Sae2) serait également impliqué dans la maturation de certaines extrémités d'ADN modifiées (Clerici *et al.*, 2005 ; Lengsfeld *et al.*, 2007). De plus, CtIP sous sa forme phosphorylée est capable d'interagir avec BRCA1 *(Breast Cancer 1)* (Yu & Chen, 2004), suggérant que cette protéine pourrait également participer à l'initiation de la résection des extrémités d'ADN (pour revue, voir Huen *et al.*, 2010). Cette étape d'initiation de la résection est essentielle dans le choix de la voie de réparation de la CDB puisqu'elle prévient les voies de recombinaison non homologue et favorise la recombinaison homologue (pour revue, voir Symington & Gautier, 2011).

La poursuite de la résection nécessite le recrutement d'autres protéines au niveau du site de la cassure : les exonucléases EXO1 et DNA2 ainsi que le complexe BLM-TOP3-RMI1 (Sgs1-Top3-Rmi1 chez la levure) (Zhu *et al.*, 2008 ; Shim *et al.*, 2010). De nombreuses études *in vitro* et *in vivo* ont permis de préciser les fonctions de ces protéines, notamment chez les Mammifères et les levures. L'exonucléase Exo1 et l'hélicase Sgs1 participent à la résection de manière indépendante puisque le double mutant *exo1 sgs1* accumule davantage de produits dont la résection est inachevée (seulement une centaine de nucléotides éliminés) par rapport aux simples mutants (Mimitou & Symington, 2008 ; Zhu *et al.*, 2008). Chez la souris, EXO1 serait également impliqué dans le recrutement de la protéine de liaison à l'ADNsb RPA *(Replication protein A)* (Schaetzlein *et al.*, 2007), une protéine jouant un rôle clé dans la résection (Cejka *et al.*, 2010a ; Niu *et al.*, 2010 ; Nimonkar *et al.*, 2011). L'hélicase BLM interagit physiquement avec EXO1 et DNA2 et stimule leurs activités nucléases (Nimonkar *et al.*, 2008 ; Nimonkar *et al.*, 2011).

L'ensemble de ces études a permis de montrer que la poursuite de la résection 5'-3' des extrémités de la cassure peut être prise en charge par deux voies parallèles (**Figure 12**). Dans la première voie, l'hélicase BLM (Sgs1) et la nucléase DNA2 interagissent physiquement et spécifiquement pour stimuler la résection. RPA favorise le déroulement de l'ADN assuré par BLM (Sgs1) ainsi que la résection 5'-3' réalisée par la DNA2. Le complexe MRN (MRX) accélère le processus en recrutant BLM (Sgs1) au niveau des extrémités. Dans la seconde voie, EXO1 dégrade l'un des brins de la double hélice grâce à son activité 5'-3' exo-nucléasique. Le complexe MRN (MRX) recrute EXO1 au niveau de la cassure et stimule son activité nucléase. BLM (Sgs1) favoriserait également cette voie en augmentant l'affinité d'EXO1 pour les extrémités d'ADN (pour revue, voir Symington & Gautier, 2011). Ces deux voies génèrent des extrémités d'ADNsb 3' sortantes de plusieurs dizaines de kilobases, nécessaires à la recombinaison homologue. Il a été rapporté qu'en absence de recombinase ou de séquence homologue, la résection pouvait se poursuivre sur plusieurs milliers de nucléotides à une vitesse d'environ 4 kilobases par heure chez la levure (Zhu *et al.*, 2008).

Chez *Arabidopsis thaliana*, des orthologues de chacune des protéines présentées précédemment ont été identifiés. AtRAD50 (Gallego *et al.*, 2001), AtMRE11 (Bundock & Hooykaas, 2002) et AtNBS1 (Waterworth *et al.*, 2007) sont les homologues des constituants du complexe MRN / MRX des Mammifères et des levures. AtCOM1 est l'homologue de CtIP/Sae2 (Uanschou *et al.*, 2007) et AtRecQl4A, l'homologue de BLM/Sgs1 (Bagherieh-Najjar *et al.*, 2003 ; Bagherieh-Najjar *et al.*, 2005). Ces différentes études ont montré que l'absence d'une de ces protéines conduit à une hypersensibilité à différents agents génotoxiques (notamment la mitomycine C) et une instabilité chromosomique en mitose ainsi qu'en méiose. La formation de foci RAD51 (la recombinase essentielle de l'invasion de brin) est très nettement réduite dans les cellules *atcom1* par rapport aux cellules sauvages, malgré la présence de CDB non réparées (Uanschou *et al.*, 2007). Ces analyses démontrent que

l'ensemble de ces protéines est impliqué dans le maintien de la stabilité du génome des plantes, et probablement dans les voies de recombinaison homologue. Deux orthologues du gène Exo1 ont également été identifiés dans le génome d'*Arabidopsis*, AtExo1A et AtExo1B (Furukawa *et al.*, 2008), ainsi qu'un orthologue du gène Dna2 (Cupp & Nielsen, 2014), mais les protéines correspondantes n'ont pas encore été caractérisées. Bien que l'étude des acteurs de la résection chez les plantes soit bien moins avancée que chez les animaux et les levures, les informations disponibles suggèrent une haute conservation de ces mécanismes au sein des Eucaryotes.

II.2. Les protéines impliquées dans la recherche d'homologie et l'invasion de brin

Review: "RAD51 mediators in eukaryotic homologous recombination"

Heïdi Serra & Charles I. White

(en préparation)

DNA double-strand breaks (DSB) are cytotoxic lesions, which arise from both exogenous (ionizing radiation, genotoxic chemicals) and endogenous sources (free radicals derived from metabolism, replicative damages) (Whitaker, 1992 ; Cox *et al.*, 2000). DSB can result in mutations, chromosomal rearrangements and/or loss of chromosomes, causing tumorigenesis or cell death. The repair of DSB by recombination is thus crucial to the maintenance of genome integrity in living organisms. Two principal mechanisms are involved in DSB repair: non-homologous end-joining and homologous recombination. The first leads to a direct joining of the break's ends, while the second implicates DNA sequence homology of the recombining molecules (for reviews, see San Filippo *et al.*, 2008 ; Waterworth *et al.*, 2011). The primary function of homologous recombination in mitotic cells is to repair single and double strand breaks but this process is also required for telomere maintenance, and consequently proliferation, in absence of telomerase. During meiosis, homologous recombination is essential to establish a physical connection between homologous chromosomes allowing their correct disjunction during the first meiotic division. Moreover, meiotic recombination creates mutations and/or new allelic combinations between genes, contributing to the generation of genetic diversity.

Among the pathways using homology for repair of DSB, Single Strand Annealing (SSA) is believed to play a particularly important role in repair of DSB flanked by direct repeat DNA sequences. SSA has been studied in yeasts, plants and animals using artificial direct repeats and could be particularly important for DSB repair in genomes that contain many repeated sequences (Lin *et al.*, 1984 ; Maryon & Carroll, 1991 ; Fishman-Lobell *et al.*, 1992). The efficiency of SSA is strongly influenced by the length of the flanking repeats, by their degree of sequence identity (Sugawara & Haber, 1992 ; Sugawara *et al.*, 1997) and by the distance separating the two repeats (Fishman-Lobell *et al.*, 1992). In the SSA model, after bidirectional 5'-3' resection of the DSB ends, the exposed complementary sequences anneal.

This step requires homologous recombination mediators as RAD52 (and Rad59 in yeast) (Ivanov *et al.*, 1996 ; Bai *et al.*, 1999 ; Liu *et al.*, 2012). Non-homologous 3' single strand DNA tails are subsequently removed by nucleases and single strand gaps filled. Strand ligation restores integrity of the DNA molecule. As DNA strand invasion and exchange are not involved, SSA is independent of RAD51 (Ivanov *et al.*, 1996 ; Stark *et al.*, 2004 ; Mansour *et al.*, 2008 ; Roth *et al.*, 2012). SSA recombination is a mutation prone pathway due to deletion of the interstitial DNA sequence lying between the repeats and one of the repeated sequences (for reviews, see Krogh & Symington, 2004 ; Heyer *et al.*, 2010).

The gene conversion homologous recombination pathway (HR) notably differs from SSA by its dependence on strand invasion catalysed by RAD51 recombinase (and/or DMC1 in meiosis). RAD51 activity has a central role in the HR process and is tightly regulated by a number of mediator proteins. In this review, we focus on the key mediators of RAD51 and propose an up-to-date synthesis of their functions in wide range of eukaryotes, including yeasts, plants, vertebrates and invertebrates. The recently described SHU complex is also reviewed and the phylogenetic relationships between components of the SHU complex and the RAD51 paralogues are discussed.

RAD51 / DMC1 recombinases and homologous recombination pathways

RAD51 is the universal recombinase of the HR pathway, highly conserved in all eukaryotes (yeast, animals and plants). RAD51 is an orthologue of the prototypic bacterial recombinase RecA and archaeal RadA (Aboussekhra *et al.*, 1992 ; Basile *et al.*, 1992 ; Shinohara *et al.*, 1992). Budding yeast *rad51* null mutants are viable but show high sensitivity to ionizing radiation and severe meiotic recombination defects (Symington, 2002a). Inactivation of RAD51 in *A. thaliana, D. melanogaster* and *C. elegans* has no effect on viability but mutants are completely sterile (Alpi *et al.*, 2003 ; Staeva-Vieira *et al.*, 2003 ; Li

et al., 2004). In vertebrates however, knockout of RAD51 results in cell inviability and early embryonic lethality (Tsuzuki *et al.*, 1996 ; Sonoda *et al.*, 1998). The severity of the *rad51* phenotype, together with the very high conservation of Rad51 protein throughout evolution, highlights the importance of RAD51/RecA activity in recombination.

RAD51 is a relatively small protein (37 kDa in human and Arabidopsis and 43 kDa in yeast) with a catalytic ATPase core region that includes Walker A/B motifs and a single-strand DNA binding domain (Shinohara *et al.*, 1992 ; Benson *et al.*, 1994 ; Sato *et al.*, 1995). RAD51 is able to nucleate on both single (ssDNA) and double strand DNA (dsDNA) *in vitro*, forming a nucleoprotein filament (NPF) (Ogawa *et al.*, 1993 ; Sung & Robberson, 1995 ; Yu *et al.*, 2001). Electron microscopic images revealed that this NPF is right-handed and composed of 18bp and approximately six RAD51 monomers per helical turn, each monomer covering three nucleotides (Ogawa *et al.*, 1993 ; Benson *et al.*, 1994 ; Yu *et al.*, 2001). The RAD51 NPF helical pitch ranges between 90 and 130 A° (Yu *et al.*, 2001 ; Conway *et al.*, 2004). ssDNA and dsDNA are thus extensively stretched within the filament, (extended in length by a factor of 1.5 compared to the B-form DNA) and dsDNA unwound (Ogawa *et al.*, 1993 ; Sung & Robberson, 1995). Subsequent real-time analyses have revealed the high flexibility and the dynamic nature of the NPF, with assembly and disassembly of RAD51 monomers regulated by many recombination mediators (Miné *et al.*, 2007 ; van der Heijden *et al.*, 2007).

The meiotic specific recombinase, DMC1 (disrupted meiotic cDNA), is present in most studied eukaryotes including yeast, vertebrates and plants (but not in *S. macrospora*, *D. melanogaster* and *C. elegans*) (Villeneuve & Hillers, 2001 ; Ramesh *et al.*, 2005 ; Malik *et al.*, 2008). DMC1 is a ~37 kDa protein, functionally, structurally and evolutionarily related to RAD51 (Bishop *et al.*, 1992 ; Story *et al.*, 1993). Ablation of DMC1 in *S. cerevisiae*, *A. thaliana* and mice produces a wide range of meiotic abnormalities that reflect the

indispensable role of DMC1 in meiotic recombination and chromosome segregation (Bishop *et al.*, 1992 ; Sehorn & Sung, 2004 ; Siaud *et al.*, 2004 ; Neale & Keeney, 2006). Human DMC1 has a weak ATPase activity, binds to ssDNA and dsDNA, and catalyses strand exchange between oligonucleotides *in vitro* (Li *et al.*, 1997 ; Masson *et al.*, 1999). The yeast DMC1 also promotes annealing of complementary ssDNA (Hong *et al.*, 2001). DMC1 exists as an octamer in solution (Passy *et al.*, 1999), and, like RAD51, is able to form right-handed helical filaments on ssDNA in an ATP-dependent manner (Masson *et al.*, 1999 ; Sehorn *et al.*, 2004 ; Sheridan *et al.*, 2008). In addition, DMC1 functions similar to RAD51 in assays for recombination and ATPase activity *in vitro* (Sehorn *et al.*, 2004 ; Bugreev *et al.*, 2005 ; Sauvageau *et al.*, 2005). DMC1 and RAD51 are both required for high rates of meiotic recombination and have overlapping, but non-identical, functions in meiosis (Shinohara *et al.*, 1997a ; Tsubouchi & Roeder, 2003 ; Bishop, 2012 ; Cloud *et al.*, 2012 ; Da Ines *et al.*, 2012). One of the key differences between both recombinases is their opposite preferential polarities of strand exchange which may explained their divergent roles in homologous recombination (Murayama *et al.*, 2011).

In the HR pathway, resection of 5'-ended DNA strands flanking the DSB generates 3' ssDNA overhangs that are rapidly bound by the single-strand binding protein, Replication Protein A (RPA). Assisted by mediator proteins, RAD51 (or DMC1) is loaded onto exposed ssDNA by displacing RPA, and forms the helical NPF (Sugiyama & Kowalczykowski, 2002) (Yu *et al.*, 2001). Presynaptic filament assembly requires ATP binding but not hydrolysis (Chi *et al.*, 2006b). Formation and maintenance of the RAD51-ssDNA NPF is required for the DNA homology search and strand exchange (Baumann *et al.*, 1996). Homology search is by random collision that involved transient nonspecific interactions with dsDNA (Bianco *et al.*, 1998). Human RAD51 facilitates homology search by a rapid A:T base-flipping mechanism (Gupta *et al.*, 1999). Once a homologous sequence is found, RAD51 promotes the formation

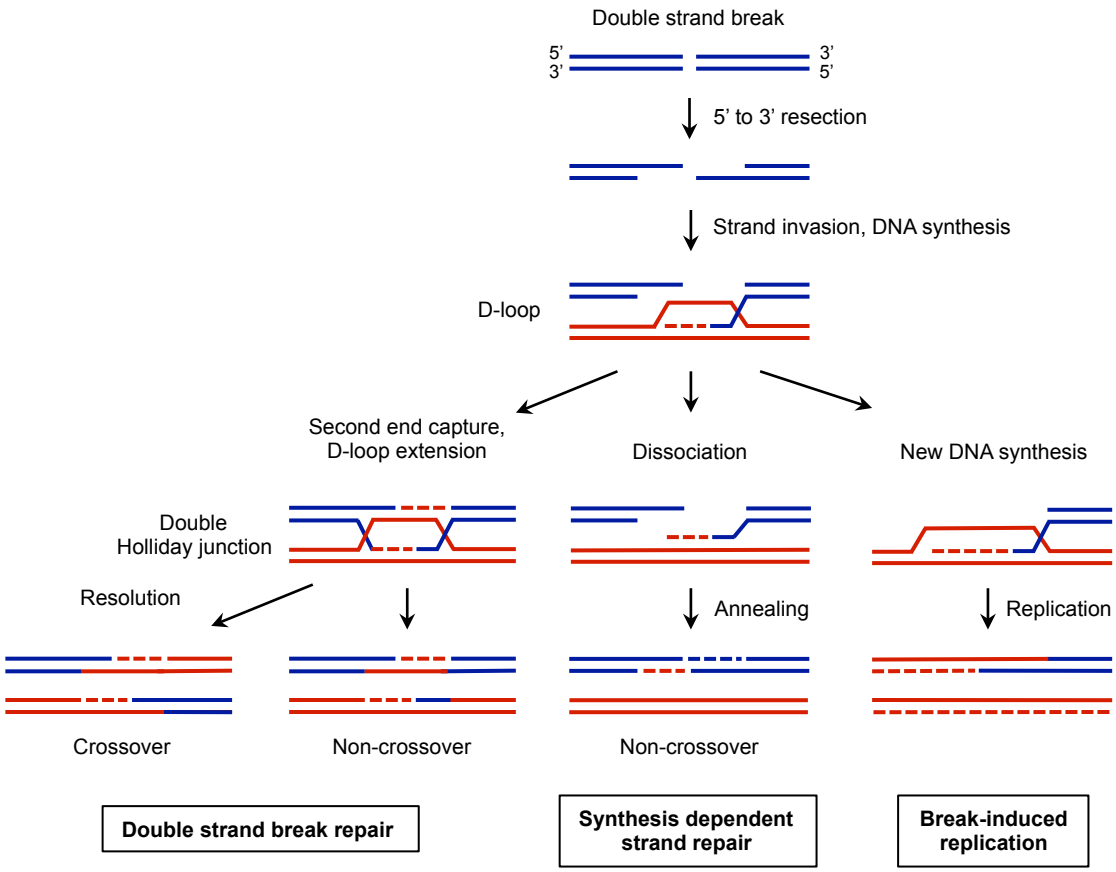


Figure 1: Models for the repair of double strand break by homologous recombination.

of a physical connection between the invading ssDNA and homologous duplex DNA template (Baumann *et al.*, 1996). This results in the generation of heteroduplex DNA with D-loop (displacement-loop) structure (**Figure 1**). Subsequent dissociation of RAD51 from DNA, correlated to ATP hydrolysis, permits to expose the invading 3'-OH end (Kowalczykowski, 1991). This extremity is used as a primer for DNA synthesis with the homologous sequence as a template.

As illustrated in Figure 1, the recombination intermediate (D-loop) can be resolved by three HR sub-pathways: DSBR, SDSA and BIR. (1) The double-strand break repair model (DSBR) involved the formation of stable double Holliday junctions (dHJ) by capture of the second-end of the DSB. DNA heteroduplex can be subsequently extended by branch migration, an ATP-dependent process driven by RAD51 recombinase (via multiple rounds of RAD51 polymerisation and dissociation) and other recombination mediators (Murayama *et al.*, 2008 ; Rossi *et al.*, 2011). The dHJs can be dissolved by the combined action of helicase/topoisomerase (only NCO products), or resolved by structure-specific endonucleases, producing crossover or non-crossover products (Szostak *et al.*, 1983 ; Schwacha & Kleckner, 1995 ; Hunter & Kleckner, 2001 ; Börner *et al.*, 2004 ; Chang *et al.*, 2005 ; Mullen *et al.*, 2005 ; Hartung *et al.*, 2008). (2) Alternatively, the invading strand can dissociate from the D-loop and anneal with the complementary 3' single strand tail on the other side of the break as described by the synthesis-dependent strand-annealing model (SDSA) (Nassif *et al.*, 1994 ; Pâques & Haber, 1999 ; Allers & Lichten, 2001 ; Puchta, 2005). The remaining gaps are filled by DNA synthesis and the nicks ligated. SDSA leads to gene conversion events not accompanied by crossovers. In somatic cells, the prevalence of gene conversion events avoids unpredictable deleterious genomic change by crossovers between ectopic sequences (Richardson *et al.*, 1998). This might be particularly important for preserving the genome stability of species containing ample amounts of repetitive DNA. During meiosis, non-

crossovers are primarily produced via SDSA processing while crossovers, prerequisite for alterations in genetic coupling arrangements, are formed by resolution of dHJ via the DSBR mechanism (Allers & Lichten, 2001 ; Hunter & Kleckner, 2001). (3) The third possibility for the resolution of the D-loop structure is the copy of the entire chromosome arm via establishment of a processive replication fork (Llorente *et al.*, 2008). This process, called break-induced replication (BIR), required leading and lagging strand DNA synthesis, in contrast to SDSA that uses only leading strand DNA synthesis (Wang *et al.*, 2004). This extensive replication primed from the invading 3' end occurs when only one DNA is available, because of the loss of the other end or in the case of the process of lengthening telomeres in telomerase-deficient cells.

REPLICATION PROTEIN A

Replication Protein A (RPA) has been identified in numerous organisms, including mammals, zebrafish, flies, nematodes, plants and budding and fission yeasts (**Table 1**). RPA is a heterotrimeric protein that binds to ssDNA with high affinity but binds poorly dsDNA and RNA (Fairman & Stillman, 1988 ; Wold & Kelly, 1988 ; Erdile *et al.*, 1990 ; Marton *et al.*, 1994 ; Sugiyama *et al.*, 1997 ; Sung, 1997a ; Hyun *et al.*, 2012). Extensive analyses have shown that RPA binds ssDNA in a sequential binding manner with a 5' to 3' polarity (De Laat *et al.*, 1998b ; Kolpashchikov *et al.*, 2000). RPA functions in a wide range of DNA processing pathways, including DNA replication, repair and recombination (see **Table 1**). In addition, a potential role of RPA in the regulation of gene transcription has been suggested in yeast and human (Singh & Samson, 1995 ; Tang *et al.*, 1996). Only its role in RAD51-dependent homologous recombination will be treated here.

RPA binds rapidly to the ssDNA resulting from the resection of the DSB extremities. RPA has higher affinity for ssDNA than Rad51 (Sugiyama *et al.*, 1997 ; Sung, 1997a) and

arrives first at DSB sites during the presynaptic phase (Wolner *et al.*, 2003). This binding to exposed ssDNA acts to protect it against degradation from endo/exo-nuclease activities and to prevent formation of DNA secondary structures that could potentially lead to inhibitory effects on Rad51 nucleoprotein filament formation (Sugiyama *et al.*, 1997). As RPA has higher affinity for ssDNA than Rad51, it inhibits Rad51 nucleoprotein complex formation by prior binding to ssDNA (Sugiyama & Kowalczykowski, 2002). This inhibition is overcome *in vivo* by RAD51 mediators such as Rad52 and Rad55-Rad57 in yeast, or BRCA2 and RAD51 paralogues in mammals (see corresponding sections below). Sugiyama and colleagues have shown that yeast Rad51 NPF formation is simultaneous with displacement of RPA from ssDNA. This displacement is initiated by a rate-limiting nucleation of Rad51 proteins onto ssDNA, followed by rapid elongation of the filament (Sugiyama & Kowalczykowski, 2002).

Once the RAD51 NPF is formed, human RPA stimulates the strand exchange activity of RAD51 by physical interaction (Baumann & West, 1997 ; Stauffer & Chazin, 2004). A similar stimulatory role was also observed in budding yeast where RPA ensures unidirectional heteroduplex extension by binding to the displaced ssDNA during the strand exchange phase (Sung, 1994 ; Eggler *et al.*, 2002 ; Van Komen *et al.*, 2002). However, during *in vitro* recombination assays, the addition of RPA to ssDNA prior to RAD51 inhibits the RAD51 reactions (Haruta *et al.*, 2006) due to inefficient displacement of RPA from ssDNA in the absence of RAD51 mediators. RPA can thus be considered as a positive regulator of HR (notably by preventing DNA secondary structure formation at all stages) and RAD51 functions, despite its apparently paradoxical role in inhibition of RAD51 NFP formation.

RAD51 PARALOGUES

The existence of RAD51/DMC1-related proteins has been described in a variety of organisms. Although clearly of common origin, these proteins show a high degree of evolutionary divergence from RAD51 as well as from each other (Thompson & Schild, 2001). Genes encoding these factors appear to be the result of gene duplication events of an ancestral RecA/RAD51 gene and the corresponding proteins have subsequently acquired new functions (Lin *et al.*, 2006).

The budding yeast *Saccharomyces cerevisiae* encodes two well-known Rad51 paralogues, Rad55 and Rad57 (Kans & Mortimer, 1991 ; Lovett, 1994). They form a stable heterodimeric complex (Sung, 1997b), which is able to integrate into the Rad51-DNA nucleofilament (Liu *et al.*, 2011a). They are positive regulators of mitotic and meiotic recombination, promoting nucleation and elongation of Rad51 NPF and stabilising it against disruption by the Srs2 antirecombinase (Liu *et al.*, 2011a). They act also in promoting of DNA strand exchange mediated by Rad51 (Sung, 1997b) (**Table 2**). Two structural and functional orthologues of Rad55 and Rad57 have been identified in the fission yeast *Schizosaccharomyces pombe*, Rhp55 and Rhp57, respectively (Khasanov *et al.*, 1999 ; Tsutsui *et al.*, 2000).

Five RAD51 paralogues have been identified in vertebrates and plants: RAD51B (RAD51L1/REC2), RAD51C (RAD51L2), RAD51D (RAD51L3), XRCC2 and XRCC3. They share 20% to 30% identity at amino acid level with RAD51 and with each other. XRCC2 and XRCC3 (X-ray Repair Cross Complementing) were first identified by their ability to complement the high sensitivity of *irs1* and *irs1SF* hamster cell lines to diverse DNA damaging agents (Tebbs *et al.*, 1995 ; Tambini *et al.*, 1997). Homology searches further identified RAD51B (Albala *et al.*, 1997 ; Rice *et al.*, 1997 ; Cartwright *et al.*, 1998a), RAD51C (Dosanjh *et al.*, 1998) and RAD51D (Cartwright *et al.*, 1998a ; Pittman *et al.*,

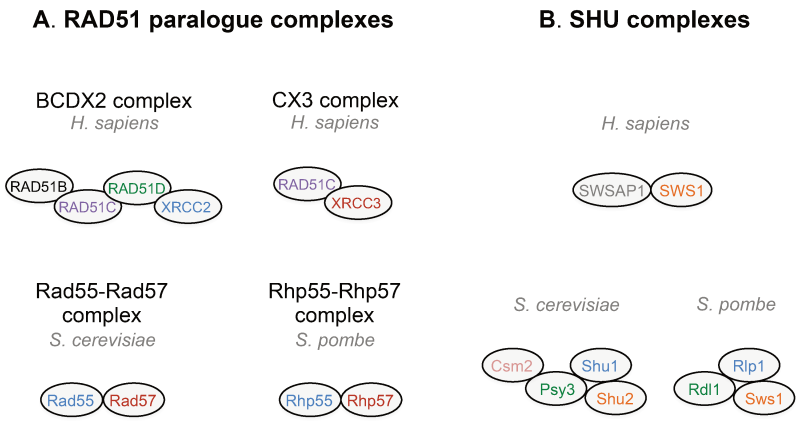


Figure 2: Composition of RAD51 paralogue complexes and SHU complexes in human and yeast

1998). The relatively low conservation of these proteins makes detailed evolutionary relationships difficult to determine with certitude, however Thacker (1999) and Tsutsui et al. (2000) have suggested that Rad55/Rhp55 are closest to XRCC2 and Rad57/Rhp57 are closest to RAD51D and XRCC3. More recent analyzes confirm this and suggest that Rad55 and Rad57 are orthologous to XRCC2 and XRCC3, respectively (Lin *et al.*, 2006).

Two-hybrid and co-immunoprecipitation studies have shown that the five RAD51 paralogues form two major complexes: RAD51B-RAD51C-RAD51D-XRCC2 (BCDX2) and RAD51C-XRCC3 (CX3), as well as RAD51B-RAD51C (BC) and RAD51D-XRCC2 (DX2) sub-complexes (Schild *et al.*, 2000 ; Masson *et al.*, 2001 ; Liu *et al.*, 2002 ; Miller *et al.*, 2002 ; Osakabe *et al.*, 2002 ; Wiese *et al.*, 2002 ; Miller *et al.*, 2004 ; Osakabe *et al.*, 2005) (**Figure 2**). No self-assembly of individual paralogues has been detected (in contrast to RAD51).

Phenotypic analyses of RAD51 paralogue mutants clearly indicate that these proteins are key mediators of the recombinational repair process in mitotic cells, but their exact roles remain to be identified (Brenneman *et al.*, 2002 ; Bleuyard & White, 2004 ; Liu *et al.*, 2004 ; Liu *et al.*, 2007 ; Badie *et al.*, 2009 ; Nagaraju *et al.*, 2009 ; Rodrigue *et al.*, 2012 ; Chun *et al.*, 2013 ; Da Ines *et al.*, 2013a). A synthesis of the properties and functions of the RAD51 paralogues are presented in Table 2. In the presynaptic phase of HR, the BCDX2 (but not the CX3 complex) is responsible for RAD51 recruitment at damage sites (Chun *et al.*, 2013). Formation and stabilization of RAD51 NPF are mediated by RAD51 paralogues, probably through counteracting disruption of the filaments by helicases (Hu *et al.*, 2007 ; Moldovan *et al.*, 2012 ; Chun *et al.*, 2013). Both CX3 (Kurumizaka *et al.*, 2001) and DX2 (Kurumizaka *et al.*, 2002) complexes catalyse homologous strand pairing *in vitro*. The human BC complex also enhances the homologous DNA pairing activity of RAD51 by suppressing the inhibitory effect of RPA (Sigurdsson *et al.*, 2001). In addition, the RAD51 paralogues are involved in regulation of gene conversion tract length (Brenneman *et al.*, 2002 ; Nagaraju *et al.*, 2009).

The association of CX3 complex with HJ in human cell extracts and the defects of HJ resolution activity in *rad51c*-deficient cells strongly suggest that CX3 is also involved in the later steps of HR in resolution of HR intermediary structures (Liu *et al.*, 2004 ; Yokoyama *et al.*, 2004 ; Kuznetsov *et al.*, 2007 ; Liu *et al.*, 2007 ; Compton *et al.*, 2010). A role of Rad51 paralogues distinct from stabilization of Rad51 nucleofilament was also reported in yeast (Mozlin *et al*., 2008 ; Fung *et al*., 2009).

Are these RAD51 paralogues involved in meiosis in addition to their key roles in somatic recombination? Consistent with the sterility phenotype of Arabidopsis *rad51c* and *xrcc3* mutants, a number of studies have demonstrated that RAD51C and XRCC3 are essential for meiotic recombination in plants, but also in vertebrates (Bleuyard & White, 2004 ; Abe *et al.*, 2005 ; Bleuyard *et al.*, 2005 ; Li *et al.*, 2005 ; Kuznetsov *et al.*, 2007 ; Liu *et al.*, 2007 ; Da Ines *et al.*, 2012). While *rad51c* and *xrcc3* mutants exhibit severe chromosomal fragmentation during meiotic prophase (Bleuyard & White, 2004 ; Li *et al.*, 2005), *xrcc2*, *rad51b* and *rad51d* mutants are however fully fertile and present no detectable meiotic defect (Bleuyard *et al.*, 2005 ; Osakabe *et al.*, 2005). These observations have led to the assumption that XRCC2, RAD51B and RAD51D play roles only in mitotic recombination (Osakabe *et al.*, 2002 ; Bleuyard *et al.*, 2005 ; Osakabe *et al.*, 2005 ; Durrant *et al.*, 2007). These three genes are however highly expressed in meiotic tissues in animals (Cartwright *et al.*, 1998a ; Cartwright *et al.*, 1998b ; Tarsounas *et al.*, 2004a) and plants (Yang *et al.*, 2011), and absence of AtXRCC2, and to a lesser extent AtRAD51B, increases rates of meiotic crossing over in Arabidopsis (Da Ines *et al.*, 2013a). The authors propose that this hyper-recombination phenotype could be due to an increase in DMC1-dependent recombination, promoted by a decrease in RAD51-dependent recombination, via the modulation of RAD51 activities by RAD51 paralogues (Da Ines *et al.*, 2013a). Although the precise roles of these proteins in

meiosis remain to be clarified, these results represent the first evidence that XRCC2 and RAD51B are involved in both mitotic and meiotic recombination.

In addition to the functions of the RAD51 paralogue complexes, individual roles for the proteins have also been reported. RAD51D is involved in telomere integrity in human cells, and appears to be the only RAD51 paralogue so involved (Tarsounas *et al.*, 2004b). The greater sensitivity of the *xrcc2 rad51b rad51d* triple mutant to bleomycin-induced DNA damage compared to corresponding single mutants (Wang *et al.*, 2014) indicates that these RAD51 paralogues have non-epistatic functions, also suggesting individual roles. In accordance to this, non-epistatic functions of Arabidopsis XRCC2, RAD51B and RAD51D have also been reported in Single Strand Annealing recombination (Serra *et al.*, 2013). Finally, a recent report shows opposing effects on cell cycle regulation of the inhibition of XRCC3 and RAD51C in HeLa cells, with inhibition of XRCC3 eliciting checkpoint defects and inhibition of RAD51C inducing G2/M cell cycle arrest (Rodrigue *et al.*, 2012).

Drosophila melanogaster encodes four RAD51-related proteins: SPN-B, SPN-D, CG2412 and CG6318 (Radford & Sekelsky, 2004). Phylogenetic analysis of RAD51 family members revealed that SPN-B is most similar to hXRCC3 (Thacker, 1999 ; Abdu *et al.*, 2003 ; Lin *et al.*, 2006) and SPN-D to hRAD51C (Abdu *et al.*, 2003 ; Lin *et al.*, 2006). Moreover, CG2412 and CG6318 appear orthologous to RAD51D and XRCC2, respectively (Sekelsky *et al.*, 2000 ; Lin *et al.*, 2006), although they are extremely divergent. Spn-B and CG2412 appear to be expressed ubiquitously, whereas Spn-D and CG6318 are expressed exclusively in the female germline (Staeva-Vieira *et al.*, 2003), suggesting meiosis-specific expression. No mutation in CG2412 and CG6318 genes have been reported and in general, very little information is available in the literature regarding the functions of the Drosophila RAD51 paralogues (**Table 2**). Staeva-Vieira et al. (2003) suggested that SPN-B, but not SPN-D, plays a role in DNA repair in somatic cells. As *spn-B* and *spn-D* mutations have identical meiotic

phenotypes (Ghabrial *et al.*, 1998 ; Abdu *et al.*, 2003), the proteins may function together in meiotic recombination. A hypothesis supported by the recent report of physical interaction between SPN-B and SPN-D (Joyce *et al.*, 2012). Further analyses will however be required to determine the functional relationships of the Drosophila RAD51 paralogues.

Contrary to all other analysed organisms, *Caenorhabditis elegans* appears to possess a unique RAD51 paralogue, RFS-1, most similar to RAD51D (Ward *et al.*, 2007). Surprisingly, RFS-1 is dispensable for mitotic and meiotic HR-mediated DSB repair (Ward *et al.*, 2007). RFS-1 is thus not a general mediator of HR-mediated repair, but performs specific roles in promoting HR repair of lesions encountered by the replication fork during S-phase (Ward *et al.*, 2007). In addition, RFS-1 is involved in telomere integrity maintenance (Yanowitz, 2008), in accordance with the implication of human RAD51D in telomere-length regulation (Tarsounas *et al.*, 2004b).

Analyses of RAD51 paralogues in various species highlight some interesting points, including differences in the severity of mutant phenotypes. Indeed, animal cells defective in any of the RAD51 paralogues are hypersensitive to DNA cross-linking agents, such as cisplatin and Mitomycin C, and show spontaneous chromosomal aberrations (Tebbs *et al.*, 1995 ; Liu *et al.*, 1998 ; Johnson *et al.*, 1999 ; Pierce *et al.*, 1999 ; Takata *et al.*, 2000 ; Takata *et al.*, 2001 ; Godthelp *et al.*, 2002). Mouse *xrcc2, rad51b, rad51c* and *rad51d* mutants are embryonic lethal (Shu *et al.*, 1999 ; Deans *et al.*, 2000 ; Pittman & Schimenti, 2000 ; Kuznetsov *et al.*, 2009). In contrast, RAD51 paralogue mutants are viable but infertile in Drosophila and the nematode *C. elegans* (Ghabrial *et al.*, 1998 ; Yanowitz, 2008). In Arabidopsis, all five RAD51 paralogue mutants grow and develop normally and only *rad51c* and *xrcc3* mutant plants are sterile due to meiotic recombination defects (Bleuyard & White, 2004 ; Bleuyard *et al.*, 2005 ; Li *et al.*, 2005). The reasons for such differences in phenotypes between organisms (such as mutant lethality/viability between vertebrates and plants) have

not to date been elucidated. A first explanation is that vertebrate RAD51 paralogues may provide additional function(s) not associated with the invertebrate, yeast and plant orthologues, but essential for organism development. It is also possible that functions of RAD51 paralogues are compensated by other protein(s) in plants and that these redundant activities do not exist in vertebrates. On the other hand, the impact on development of cell death due to DNA damage differs in vertebrates and plants. Indeed, death of individual cell in plants does not translate simply to death of the plant, but is compensated by division of other cell and at most lead to a temporary slowing of the plant's growth. Cell death potentially induced by the absence of RAD51 paralogues in the vertebrate embryo can cause developmental arrest and/or lethal abnormalities. Due to this inviability of vertebrates lacking one of the RAD51 paralogues (Shu *et al.*, 1999 ; Deans *et al.*, 2000 ; Pittman & Schimenti, 2000 ; Kuznetsov *et al.*, 2009), plants thus provide an interesting model to study functions of the RAD51 paralogues.

Comparison of RAD51 paralogue functions between eukaryotes both confirms their high conservation and, highlights interesting functional divergences. For example, Arabidopsis XRCC2 is clearly involved in RAD51-independent SSA recombination (Serra *et al.*, 2013) whereas its yeast counterpart, Rad55, has an inhibitory effect on SSA via promotion of HR (Godin *et al.*, 2013). Concerning the requirement of RAD51 paralogues in formation and stabilization of RAD51 NPF, some differences have been reported between organisms. Yeast Rad55 and Rad57 are required for the formation of RAD51 foci in meiosis, but only partially in mitotic cells (Gasior *et al.*, 1998 ; Lisby *et al.*, 2004). Chinese hamster ovary (CHO) and mouse embryonic fibroblast (MEF) cells defective in any of the five RAD51 paralogues exhibit defects in the formation of DNA damage-induced RAD51 foci (Bishop *et al.*, 1998 ; Tarsounas *et al.*, 2004a ; Smiraldo *et al.*, 2005 ; Van Veelen *et al.*, 2005). However, the requirement of RAD51 paralogues in RAD51 focus formation is by-passed in

the context of spontaneous S-phase replication-associated RAD51 foci in CHO (Tarsounas *et al.*, 2004a). Experiments in chicken DT40 cells showed that mutation of any of the RAD51 paralogues significantly reduces, but doesn't eliminate the formation of RAD51 foci (Takata *et al.*, 2000 ; Takata *et al.*, 2001 ; Yonetani *et al.*, 2005 ; Qing *et al.*, 2011). Recent work in plants has revealed that Arabidopsis RAD51B, RAD51D and XRCC2 are not required for the formation of radio-induced RAD51 foci in somatic cells (Da Ines *et al.*, 2013a). Taken together, these results highlight an apparent variability in the need for the RAD51 paralogues in recruitment/stabilization of RAD51 at DNA damage sites. Given the transient presence of the foci and that the kinetics of focus appearance/disappearance can be modified in mutants, this may however be an artefact due to the experimental conditions. In agreement with this, it has recently been demonstrated that mutation of XRCC2 leads to a delay (not absence) in RAD51 focus formation in hamster cells (Tambini *et al.*, 2010). It is however also possible that these data reflect real differences in the requirement for RAD51 paralogues in RAD51 NPF formation/stabilization between yeasts, animals and plants.

SHU COMPLEX

Considerable interest is being given to four additional RAD51 mediators characterised in *Saccharomyces cerevisiae*: Shu1, Shu2, Psy3 and Csm2, components of the SHU (or PCSS) complex (Shor *et al.*, 2005). Shu1 and Shu2 (suppressor of sgs1 hydroxyurea sensitivity) were first identified by a screen for suppressors of the slow-growth phenotype of yeast topoisomerase III (*top3*) mutants (Shor *et al.*, 2005). Psy3 and Csm2 were initially discovered by genome-wide screens for mutants sensitive to platinium (Wu *et al.*, 2004) or affecting chromosome segregation in meiosis (Rabitsch *et al.*, 2001), respectively. Yeast two-hybrid experiments have identified the physical interactions between these proteins (Ito *et al.*, 2001 ; Shor *et al.*, 2005 ; Ball *et al.*, 2009), and thus permit specification of the organisation

of the complex (see **Figure 2**): Psy3 would be the core protein, the sole that simultaneously interacts with the three other components. This stable complex is able to form a nucleofilament by binding non-specifically to DNA, like Rad51 (Tao *et al.*, 2012). The key function of the SHU complex is to promote RAD51 NPF formation by inhibiting Srs2 anti-recombinase recruitment at inducible DSB sites (Bernstein *et al.*, 2011). The SHU complex could directly inhibit the action of Srs2 through its interaction with Shu2 (Ito *et al.*, 2001), like Rad55-Rad57, and/or be a stabilizer of RAD51 filament. This complex clearly promotes HR while suppressing error-prone DNA repair (Shor *et al.*, 2005 ; Mankouri *et al.*, 2007 ; Ball *et al.*, 2009) and given that its absence leads to sensitivity to replication blocking agents, it may have a specialized role in HR during replicative stress (Ball *et al.*, 2009 ; Choi *et al.*, 2010 ; Xu *et al.*, 2013). Recent reports have also revealed a crucial role in meiotic recombination in mediating assembly of Rad51 NPF (Sasanuma *et al.*, 2013) and in promoting the establishment of homolog bias during meiotic HR (Hong & Kim, 2013). Available data about SHU complex functions are summarized in Table 3. Further work will however be needed to fully establish the roles of the SHU proteins.

Some components of the SHU complex are evolutionally conserved in fission yeast and in human: Rlp1, Sws1 and Rdl1 are the three identified components of *Schizosaccharomyces pombe* SHU complex and SWS1 and SWSAP1 are members of human SHU complex (Martín *et al.*, 2006 ; Liu *et al.*, 2011b ; Sasanuma *et al.*, 2013). These proteins form stable complexes *in vivo*: Rlp1-Sws1-Rdl1 in fission yeast and SWS1-SWSAP1 in human (**Figure 2**) and have an evolutionarily conserved function required for efficient HR repair (Martín *et al.*, 2006 ; Liu *et al.*, 2011b) (**Table 3**). Liu et al. (2001) suggest than human SHU complex is a non-essential HR accessory factor, which affects the efficiency and/or timing of HR.

What are the phylogenetic relationships between human SHU complex components, their yeast counterparts and well-known RAD51 paralogues? Data from literature on this subject show some discrepancy. Shu1 and Psy3 would be RAD51 paralogues (Martín *et al.*, 2006). Sequence homology analyses indicate that budding yeast Shu1, Shu2, Psy3 and fission yeast Rlp1, Sws1, Rdl1 correspond to human XRCC2, SWS1 and RAD51D, respectively (Khasanov *et al.*, 2004 ; Martín *et al.*, 2006). Rdl1 and Psy3 are significantly similar whereas Rlp1 shares no obvious sequence similarities to Shu1 (or Csm2) (Martín *et al.*, 2006). No human or fission yeast protein related to Csm2 has yet been found. Although the yeast proteins are associated as a complex, XRCC2, RAD51D (or other RAD51 paralogues) were not detected in the mass spectrometry analysis of hSWS1-containing complexes (from human 293T cells) (Liu *et al.*, 2011b). This screen did however identify SWSAP1 (SWS associated protein 1), a new interacting partner which has similarity to RadA, a RecA family protein in *archaea* (Liu *et al.*, 2011b). Divergences of protein domains and sequences have thus frustrated efforts to identify reliable phylogenetic correlations between the components of SHU complex and RAD51 paralogues. The recent resolution of the crystal structures of Psy3 and Csm2 have revealed that these proteins share a similar structure with Rad51 (She *et al.*, 2012 ; Tao *et al.*, 2012) and that Psy3-Csm2 sub-complex is a structural mimic of the Rad51-dimer (Sasanuma *et al.*, 2013). These results suggest that the structure (and functions), but not the sequence, of these proteins has been conserved.

The existence of several RAD51-like protein complexes, like SWS1-SWSAP1, BCDX2 and CX3 in human, raises the question of their functional relationships. Many lines of evidence argue for an interconnected and coordinated action of the SHU complex with the other RAD51 mediators. Rad55 physically interacts with SHU complex via Csm2 protein (Godin *et al.*, 2013). *csm2* and *psy3* are epistatic to *rad55* (Godin *et al.*, 2013 ; Xu *et al.*, 2013) and these two proteins are needed for efficient recruitment of Rad55 to DNA repair foci

after DNA damage (Godin *et al.*, 2013). Shu2 favours Rad51 focus formation (Bernstein *et al.*, 2011), but also Rad52 focus formation (Shor *et al.*, 2005). In addition, Shor *et al.* (2005) highlighted an epistatic relationship between *rad52* and *shu* mutations, suggesting that Shu proteins function together with Rad52. Human SWS1 is able to associate with RAD51D and XRCC3, and SWSAP1 interacts with RAD51B, RAD51C, RAD51D and XRCC3 (Martín *et al.*, 2006 ; Liu *et al.*, 2011b). Moreover, chicken SWS1 requires BRCA2 to contribute to HR (Qing *et al.*, 2011). Taken together, these data underscore the interconnected roles of mediators and the importance of controlling HR repair.

In view of the high conservation of RAD51 mediators, especially RAD51 paralogues, between animals and plants, it is very likely that the components of the SHU complex also exists in other mammals and plants.

RAD52 AND RAD59

The RAD52 protein has been identified in most eukaryotic organisms, including yeasts, vertebrates and recently in plants (Adzuma *et al.*, 1984 ; Bezzubova *et al.*, 1993 ; Muris *et al.*, 1994 ; Samach *et al.*, 2011), but appears to be absent in invertebrates (Lok & Powell, 2012). Structure and biochemical activities of RAD52 proteins from different species are very similar. Electron microscopic evidence indicates that both yeast and human RAD52 form oligomeric ring structures (Shinohara *et al.*, 1998 ; Stasiak *et al.*, 2000 ; Ranatunga *et al.*, 2001). RAD52 proteins bind ssDNA and dsDNA in a sequence-independent manner and stimulate annealing of complementary ssDNA *in vitro* (Mortensen *et al.*, 1996 ; Reddy *et al.*, 1997 ; Shinohara *et al.*, 1998 ; Sugiyama *et al.*, 1998). RAD52 is also able to interact directly with the Replicative Protein A (RPA) (Hays *et al.*, 1998 ; Sugiyama *et al.*, 1998 ; Jackson *et al.*, 2002 ; van den Bosch *et al.*, 2002). It accumulates at DNA damage sites, forms discrete nuclear foci and co-localizes with RAD51 *in vivo* (Liu *et al.*, 1999 ; Liu & Maizels, 2000 ; Lisby *et al.*, 2001 ; Essers *et al.*, 2002).

Budding yeast Rad52 plays an essential role in DSB repair and HR with two main functions which both use RPA-coated ssDNA as a substrate: it facilitates the Rad51 NPF formation in the presence of RPA and promotes DNA annealing (Sung, 1997a ; New *et al.*, 1998 ; Shinohara *et al.*, 1998 ; Sugiyama *et al.*, 1998 ; Sugiyama & Kowalczykowski, 2002) (**Table 1**). Rad52 mediator activity is crucial to overcome the inhibitory action of RPA (when RPA is added prior to Rad51). Rad51 recruitment is dependent on Rad52 during both mitotic and meiotic DSB repair (Gasior *et al.*, 1998 ; Sugawara *et al.*, 2003 ; Wolner *et al.*, 2003 ; Fung *et al.*, 2009). Yeast Rad52 is able to interact directly with Rad51 (Shinohara *et al.*, 1992 ; Milne & Weaver, 1993 ; van den Bosch *et al.*, 2002) and both recruitment and nucleation of Rad51 onto RPA-coated DNA requires Rad51-Rad52 interaction (Shinohara *et al.*, 1998 ; Krejci *et al.*, 2002). Moreover, the strand-annealing activity of Rad52 facilitates the second-end capture leading the formation of a HJ during SDSA pathway (Wu *et al.*, 2008 ; Nimonkar *et al.*, 2009). In addition to its essential role in HR, yeast Rad52 has also been implicated in RAD51-independent events, such as BIR (McEachern & Haber, 2006) and SSA (Sugawara & Haber, 1992), where its DNA annealing activity is required for annealing of the two tandemly repeated ssDNA sequences covered by RPA. *Schizosaccharomyces pombe* possess two homologues of *S. cerevisiae* Rad52, Rad22A+ and Rad22B+, which share with Rad52 similar functions in HR and SSA (Ostermann *et al.*, 1993 ; Kim *et al.*, 2000 ; van den Bosch *et al.*, 2001) (**Table 1**). While Rad22A+ plays a more important role during vegetative growth (van den Bosch *et al.*, 2001), Rad22B+ would essentially act during meiosis (Octobre *et al.*, 2008).

Rad59 was identified in *S. cerevisiae* as a Rad52 protein homologue (Bai & Symington, 1996), but surprisingly, no Rad59 homologue has been found in fission yeast. It shares sequence similarity with the conserved N-terminal domain of Rad52 protein but lacks the C-terminal Rad51-interacting domain (Feng *et al.*, 2007). Rad52 and Rad59 bind ssDNA and can mediate annealing of complementary ssDNA, but only Rad52 interacts with Rad51

and RPA (Mortensen *et al.*, 1996 ; Petukhova *et al.*, 1999a ; Davis & Symington, 2001 ; Wu *et al.*, 2006). *rad59* displays subtle epistasis interactions with *rad52* in a variety of DSB repair events, suggesting that the two proteins cooperate in multiple contexts (Feng *et al.*, 2007 ; Manthey & Bailis, 2010). Mutation of Rad59 can in addition be partially complemented by overexpression of Rad52, suggesting that the functions of both proteins overlap (Bai & Symington, 1996). Thus, Rad59 possesses distinct Rad52-dependent and -independent functions (Pannunzio *et al.*, 2012). Rad59 and Rad52 interact *in vivo* (Davis & Symington, 2001) and Rad59 plays a critical role in localization of Rad52 to DSB (Pannunzio *et al.*, 2012). Unlike yeast Rad52, Rad59 is dispensable in Rad51-dependent HR pathways but is a crucial factor of the RAD51-independent SSA pathway (Bai & Symington, 1996 ; Jablonovich *et al.*, 1999 ; Petukhova *et al.*, 1999a ; Sugawara *et al.*, 2000 ; Davis & Symington, 2001).

Although Rad52 plays an essential role in HR in budding yeast, it plays a more modest role in vertebrates, where additional proteins like BRCA2 have evolved to perform similar functions. Indeed, RAD52 knockout in mouse embryonic cells and in chicken DT40 cells does not cause an apparent sensitivity to IR or DSB-causing chemical agents (Rijkers *et al.*, 1998 ; Yamaguchi-Iwai *et al.*, 1998). Despite structural and some biochemical similarity between human and yeast RAD52, the human protein appears functionally similar to the yeast Rad59 protein. Human RAD52 does not appear to have a recombination mediator activity in reconstituted biochemical assays (Jensen *et al.*, 2010), while it conserves a strand annealing activity (Van Dyck *et al.*, 2001 ; Singleton *et al.*, 2002). A recent report has shown that human RAD52 acts in parallel to BRCA2 and that its inactivation is lethal in BRCA2 deficient cells (Feng *et al.*, 2011). These observations argue for a model in which RAD52 functions in an alternative pathway to BRCA2 (Liu & Heyer, 2011) which is suggested to support the proliferation of human tumour cells deficient in BRCA2 *via* mediation of RAD51

functions (Feng *et al.*, 2011). RAD52 however also has a function in the late stages of DSB repair at stalled or collapsed replication forks that does not appear to be shared by BRCA2 (Fujimori *et al.*, 2001). The inviability and extensive chromosomal breaks of *rad52 xrcc3* double knockout DT40 cells implies that RAD52 might function as a recombination mediator in conjunction with one or a combination of the RAD51 paralogues (Fujimori *et al.*, 2001 ; Liu & Heyer, 2011).

The question of whether plants possess a RAD52 homologue has long left open (Iyer *et al.*, 2002 ; Mortensen *et al.*, 2009). In 2011, Samach et al. reported that RAD52 homologues are present in all plants whose genomes have undergone extensive sequencing. The RAD52 gene apparently underwent very early duplication, followed by later duplication in specific lineages, during the evolution of higher plants (Samach *et al.*, 2011). Plant RAD52 proteins share high sequence similarity to the known RAD52 proteins (Samach *et al.*, 2011), suggesting similar function for these two families. Two RAD52 homologues have been identified in *Arabidopsis thaliana*: RAD52-1 and RAD52-2. Mutants or RNA interference lines defective in the expression of one or both of these showed mildly reduced fertility, sensitivity to MMC and decreased levels of intrachromosomal recombination compared to the wild type (Samach *et al.*, 2011). In addition, Arabidopsis RAD52-1A partially complemented the yeast *rad52* mutant. These functional assays provide evidence for the role of Arabidopsis RAD52 in DNA recombination and repair and suggest conserved functions of RAD52 in eukaryotes. Given the presence of BRCA2 (see next section) and the moderate effect of plant rad52 mutants on HR, the plant RAD52 genes appear to more closely resemble human RAD52 (Samach *et al.*, 2011). Further work will however be required to identify the mechanisms through which the diverse RAD52 proteins affect DNA recombination in plants and animals.

BRCA1 AND BRCA2

Despite their absence in budding and fission yeasts, the BRCA1 and BRCA2 proteins appear relatively well conserved in eukaryotes (Lo *et al.*, 2003 ; Trapp *et al.*, 2011 ; Lok & Powell, 2012) (**Table 4**). However, no orthologue of BRCA1 has yet been identified in flies and zebrafish (Meetei *et al.*, 2004 ; Howe *et al.*, 2013). These two proteins are encoded by tumour suppressor genes in mammals, with hereditary mutations in the *brca1* or *brca2* genes being associated with high breast cancer susceptibility and increased risk of ovarian, pancreatic, stomach, laryngeal, fallopian tube and prostate cancer (Roy *et al.*, 2012). Extensive evidence shows that BRCA1 and BRCA2 are key actors in maintaining genome stability, especially by promoting efficient and precise repair of DSB. Many outstanding reviews on human BRCA1 and BRCA2 proteins have been written (Gudmundsdottir & Ashworth, 2006 ; Roy *et al.*, 2012 ; Caestecker & Van de Walle, 2013) and only a brief overview, including more recently described aspects, will be given here. We will take care to include less well-known data from invertebrates and plants.

The BRCA1 protein is an E3 ubiquitin ligase which catalyses protein ubiquitylation in *vitro* (Hashizume *et al.*, 2001 ; Ruffner *et al.*, 2001). BRCA1 has been implicated in interactions with more than 20 proteins (Welcsh & King, 2001), including RAD51 (Scully *et al.*, 1997b), RAD50 (Zhong *et al.*, 1999) and BRCA2 (Chen *et al.*, 1998a). *In vivo*, BRCA1 associates and colocalizes with RAD51 in nuclear foci in mitotic cells (Scully *et al.*, 1997b) and these foci also contain BRCA2 and BARD1 (BRCA1-binding protein) (Jin *et al.*, 1997 ; Scully *et al.*, 1997a ; Chen *et al.*, 1998a). Subsequent studies have revealed that BRCA1 is required for RAD51 foci formation in mouse cells (Bhattacharyya *et al.*, 2000). Most homozygous *brca1* mutations lead to embryonic lethality in mice (Hakem *et al.*, 1996 ; Deng & Scott, 2000 ; Evers & Jonkers, 2006), clearly showing the biological importance of this protein. In *C. elegans*, inactivation of BRC-1 (the nematode BRCA1 orthologue) results in

high incidence of males due to nondisjunction of X chromosomes and elevated levels of germ cell death (Boulton *et al.*, 2004). In addition to high sensitivity to IR and chromosomal instability (Shen *et al.*, 1998 ; Xu *et al.*, 1999), *brca1* mouse embryonic stem cells exhibit impaired DSB repair by HR (Moynahan *et al.*, 1999 ; Moynahan *et al.*, 2001). As DSB repair by SSA is also reduced in *brca1* deficient cells, BRCA1 may act before the branch point of HR and SSA (Stark *et al.*, 2004). In accordance with this, BRCA1 promotes CtlP-mediated 5'-end resection of DSB in human cells (Yun & Hiom, 2009). BRCA1 does not seem to be involved in End Joining recombination pathways, although data on this subject are conflicting (Moynahan *et al.*, 1999 ; Baldeyron *et al.*, 2002 ; Zhong *et al.*, 2002 ; Stark *et al.*, 2004 ; Wang *et al.*, 2006 ; Zhuang *et al.*, 2006 ; Yun & Hiom, 2009). In addition to its role in DSB repair, BRCA1 has also been connected to various cellular processes in response to DNA damage in animal cells, such as DNA damage signalling, chromatin remodelling and checkpoint control. Roles for human BRCA1 in the regulation of telomere length (Ballal *et al.*, 2009) and in meiotic sex chromosome inactivation during spermatogenesis (Turner, 2007) have also been described. It is however not excluded that the action of BRCA1 in these processes is indirect. For more details concerning these other functions, see Table 4 and specific reviews (Gudmundsdottir & Ashworth, 2006 ; Roy *et al.*, 2012 ; Caestecker & Van de Walle, 2013).

Putative homologues of BRCA1 have been identified in almost all sequenced plant genomes (Trapp *et al.*, 2011). Plant BRCA1 differs from animal BRCA1 by the presence of the PHD domain (plant homeodomain). PHD domains were previously shown to bind lysine 4 trimethylated histone H3 (H3K4me3), which can act as a dynamic signal for transcriptionally active genes and mark initiation sites for meiotic recombination (Santos-Rosa *et al.*, 2002 ; Borde *et al.*, 2009). Although an exciting possibility, such a role of the plant BRCA1 PHD domain has not however been tested to date. Arabidopsis BRCA1 shows an overall identity of

approximately 20% at the amino acid level with its human counterpart and the BRCT and RING domains are conserved (Lafarge & Montané, 2003). It is thus likely that this RING domain also exhibits E3-ligase activity in Arabidopsis. Interestingly, homozygous *brca1* Arabidopsis plants show no developmental abnormalities (Lafarge & Montané, 2003 ; Reidt *et al.*, 2006). Similarly, BRCA1 is not essential for meiosis as the amount of viable seeds and pollen are similar in mutants and wild-type plants (Reidt *et al.*, 2006), although possible minor functions may exist. Arabidopsis *brca1* mutants do however have defects in HR, especially prominent after induction of DSB by bleomycin (Block-Schmidt *et al.*, 2011). Also, Reidt et al (2006) have shown that BRCA1 is involved in DNA crosslink repair, in accordance with data in human (Westermark *et al.*, 2003). Taken together, these results indicate that animal and plant BRCA1 are required for efficient DSB repair by HR in somatic cells and the existence of plant-specific domain could suggest more extensive roles in plants.

BRCA2 was first identified in a screen for high-penetrance germline mutations associated with breast cancer susceptibility (Wooster *et al.*, 1995). Orthologues of BRCA2 have subsequently been identified in various organisms, including mouse, chicken, zebrafish, Drosophila, *C. elegans* and some plant species (**Table 4**) (Sharan & Bradley, 1997 ; Warren *et al.*, 2003 ; Siaud *et al.*, 2004 ; Martin *et al.*, 2005 ; Titus *et al.*, 2006 ; Klovstad *et al.*, 2008). Comparison of BRCA1 and BRCA2 proteins indicates that they are unrelated proteins (for a review, see Powell & Kachnic, 2003). Indeed, while BRCA1 is characterized by the BRCT motif and the RING domain (with E3 ubiquitin ligase structure), BRCA2 possess a ssDNA binding domain and several BRC peptide motifs (crucial for RAD51 binding). *In vivo* BRCA2-RAD51 interaction have been reported in all analysed animals (Wong *et al.*, 1997 ; Chen *et al.*, 1998b ; Martin *et al.*, 2005 ; Brough *et al.*, 2008). Further analyses precise that one human BRCA2 molecule can bind 6 molecules of RAD51 (Liu *et al.*, 2010). BRCA2 seems to be required for the correct localization of RAD51 and DMC1 recombinases to DSB

in mice (Sharan *et al.*, 2004). According to this, numerous studies have demonstrated an early role of BRCA2 in HR by promoting the assembly of RAD51 (and probably DMC1) onto RPA-coated ssDNA and stabilization of RAD51 NPF (Xia *et al.*, 2006 ; Petalcorin *et al.*, 2007 ; Jensen *et al.*, 2010 ; Liu *et al.*, 2010 ; Thorslund *et al.*, 2010). A recent study revealed that human BRCA2 is also a key regulator of the extension step after strand invasion at replication-dependent DSB (Buisson *et al.*, 2014), therefore BRCA2 would involved not only in stimulation of RAD51 activities but also in subsequent DNA synthesis. Consistent with its key mediator role in the core mechanism of HR, inactivation of *brca2* in mice results in an early embryonic lethality associated with chromosomal instability (Hakem *et al.*, 1998 ; Evers & Jonkers, 2006).

In addition to its interaction with DMC1, numerous data support a role of BRCA2 in meiotic recombination: it forms foci associated with meiotic chromosomes during synaptonemal complex formation (Chen *et al.*, 1998a), it is involved in ovarian development and tumorigenesis in zebrafish reproductive tissues (Shive *et al.*, 2010) and the expression of a truncated BRCA2 in mouse leads to a failure in spermatogenesis (Connor *et al.*, 1997). In addition, Ko et al. have shown that BRC-2, the BRCA2 homologue in *C. elegans*, plays an essential role in chromosome integrity of germ cells and is crucial in meiotic progression (Ko *et al.*, 2008). Drosophila BRCA2 has also been connected to efficient activation of the meiotic recombination checkpoint (Klovstad *et al.*, 2008). The potential function of BRCA2 in SSA is not clear: it seems to supress this pathway in mouse (Stark *et al.*, 2004) while CeBRC-2 promotes the SSA pathway in nematode cells (Petalcorin *et al.*, 2006). One possibility is that CeBRC-2 may have replaced the role of vertebrate RAD52 (missing from *C. elegans)* in SSA.

Two BRCA2 homologues have been identified in the plant *Arabidopsis thaliana*: BRCA2A (or BRCA2(IV), localised in the subtelomeric region of chromosome IV) and BRCA2B (BRCA2(V), on chromosome V) (Siaud *et al.*, 2004). Arabidopsis is the only

species reported to have two homologues of the BRCA2 gene. Although much research has been conducted on Arabidopsis BRCA2 proteins, no data are available on the BRCA2 homologues in other plants. Like mammalian BRCA2, both Arabidopsis BRCA2 proteins are able to interact with AtRAD51 and AtDMC1 in yeast two-hybrid assays, suggesting a conserved role of plant BRCA2 in mitosis and meiosis (Siaud *et al.*, 2004). Silencing of the BRCA2 genes specifically in meiosis (to prevent potential somatic phenotypes) results in a partial sterility phenotype, associated with dramatic meiotic alterations including the absence of pairing, synapsis and bivalent formation, defects in anaphase I and II and chromosome fragmentation (Siaud *et al.*, 2004). This *brca2* mutant phenotype is suppressed in a *spo11-1* background (Siaud *et al.*, 2004), demonstrating that BRCA2 function is dependent upon meiotic DSB. More recently, Seeliger et al. (2012) have generated simple and double *brca2* mutant plants and showed that they are viable throughout somatic growth and that only the double mutant exhibits sterility phenotype. Therefore, it appears that the role of the two Arabidopsis BRCA2 proteins in meiosis in redundant. The double mutants also showed hypersensitivity to MMC and a dramatic reduction in somatic HR frequency, especially after DSB induction (Seeliger *et al.*, 2012). Taken together, these results demonstrate an important role of BRCA2 in both somatic and meiotic RAD51/DMC1-dependent HR in plants. In addition to evolutionarily conserved functions in DNA repair and recombination, recent research has surprisingly revealed a function of Arabidopsis BRCA2 in plant defense mechanisms. BRCA2A, but not BRCA2B, specifically regulates pathogenesis-related gene transcription in response to salicylic acid signalling during plant immune responses (Wang *et al.*, 2010). This discovery challenges the idea that BRCA2 only a specific role in mediating HR - like BRCA1, Arabidopsis BRCA2 appears to be involved in various cellular processes.

The normal vegetative development of plants carrying homozygous *brca1* or *brca2* mutations (in comparison to the death at a very early developmental stage observed in

mammals) highlights the spectacular resistance of plant development to DNA damage in, the absence of key DNA metabolism proteins. Thanks to this capacity, plants offer a chance to study the function of these proteins during the complete life cycle of a multicellular eukaryote.

RAD54 AND RAD54B

RAD54 is a highly conserved gene: RAD54 homologues have been identified in most eukaryotes including human, mice, chicken, zebrafish, Drosophila, Arabidopsis and yeast (**Table 1**) (Kanaar *et al.*, 1996 ; Muris *et al.*, 1996 ; Bezzubova *et al.*, 1997 ; Kooistra *et al.*, 1997 ; Thoma *et al.*, 2005 ; Shaked *et al.*, 2006). The Drosophila homologue of RAD54 is termed *okra* (Ghabrial *et al.*, 1998). RAD54 is a member of the SWI2/SNF2 protein family and exhibits, as do the other members of this family, a dsDNA-dependent ATPase, DNA translocase, DNA supercoiling and chromatin remodeling activities (for reviews, see Heyer *et al.*, 2006 ; Mazin *et al.*, 2010 ; Ceballos & Heyer, 2011). RAD54 is an ATP-dependent motor protein that tracks along dsDNA but is unable to catalyse the strand displacement reactions typical of DNA helicases (Flaus *et al.*, 2006). Its ATPase activity requires dsDNA and is not supported by ssDNA (Swagemakers *et al.*, 1998), whereas ATPase activity of classical DNA helicases is stimulated by ssDNA (for a review, see Singleton *et al.*, 2007). Single molecule experiments directly visualized *S. cerevisiae* Rad54 translocation on dsDNA, demonstrating highly processive movement at 300 bp/second (Amitani *et al.*, 2006). dsDNA tracking by RAD54 is associated with topological changes in the DNA, forming positive and negative supercoils in topologically constrained DNA molecules (Tan *et al.*, 1999 ; Van Komen *et al.*, 2000 ; Ristic *et al.*, 2001 ; Jaskelioff *et al.*, 2003). Extensive biochemical studies have demonstrated that the presence of RAD51-DNA filament significantly enhances ATP hydrolysis activity and Rad54-induced topological changes of yeast Rad54 (Mazin *et al.*,

2000 ; Van Komen *et al.*, 2000 ; Kiianitsa *et al.*, 2002), suggesting that Rad54 functions in concert with RAD51 *in vivo*.

Similar to *rad51* mutants, *S. cerevisiae rad54* mutants are extremely sensitive to ionizing radiation but also to cross-linking (MMC, cisplatinium) or alkylating agents (MMS), and other agents that are able of causing DSB (Krogh & Symington, 2004). Yeast *rad54*-deficient cells are defective in intra- and inter-chromosomal gene conversion (Shinohara *et al.*, 1997b). In mice, inactivation of RAD54 is viable (in contrast to the *rad51* mutant) but animals exhibit an hypersensitivity to DNA cross-linking agents at all development stages and to IR only at embryonic stages, due to rescue by End-Joining repair in adult stage (Essers *et al.*, 1997 ; De Silva *et al.*, 2000 ; Essers *et al.*, 2000 ; Essers *et al.*, 2002). The impact of absence of RAD54 on fertility differs depending on the organism studied: *rad54* mutant mice and Arabidopsis are fertile (Essers *et al.*, 1997 ; Osakabe *et al.*, 2006) and *rad54* yeasts are able to produce some viable spores (Muris *et al.*, 1997), while *rad54* Drosophila females are sterile (Kooistra *et al.*, 1997) (**Table 1**). Taken together, these data suggest that RAD54 is involved in DSB repair, but does not play an essential role in meiosis, at least in mammals, plants and yeasts. In mammalian cells, RAD54 proteins accumulate at DNA damage sites and colocalize with RAD51 foci following IR-treatment (Tan *et al.*, 1999 ; Essers *et al.*, 2002). Although RAD54 focus formation is dependent on Rad51, cytological studies in yeast, chicken DT40 cells and mouse ES cells suggest that RAD54 is not required for the formation of Rad51 foci, indicating that Rad54 acts downstream of Rad51 (Shinohara *et al.*, 2000 ; Takata *et al.*, 2000 ; Lisby *et al.*, 2004 ; Miyazaki *et al.*, 2004 ; Van Veelen *et al.*, 2005).

Many studies, conducted primarily in mammals and yeast, have demonstrated that RAD54 acts at all stages of homologous recombination, although some of its roles are not yet well understood. *In vitro* assays have showed that RAD54 facilitates RAD51 binding to ssDNA in the presence of RPA and stabilizes the RAD51 NPF without requiring RAD54

ATPase activity (Mazin *et al.*, 2003 ; Wolner *et al.*, 2003). However, the significance of these biochemical studies is unclear because Rad51 focus formation in response to DSB does not seem to be dependent on Rad54 (Shinohara *et al.*, 2000 ; Takata *et al.*, 2000 ; Lisby *et al.*, 2004 ; Miyazaki *et al.*, 2004 ; Van Veelen *et al.*, 2005). RAD54 is involved in stimulation of RAD51-mediated three-strand exchange activity and D-loop formation. This RAD54 function was first demonstrated with recombinant yeast proteins (Petukhova *et al.*, 1998) and then extended to all the examined systems (**Table 1**) (Alexiadis & Kadonaga, 2002 ; Sigurdsson *et al.*, 2002 ; Mazina & Mazin, 2004). The stimulation depends on the ATPase activity of RAD54 and required species-specific protein interactions between the RAD51 and RAD54 proteins (Jiang *et al.*, 1996 ; Clever *et al.*, 1997 ; Golub *et al.*, 1997 ; Petukhova *et al.*, 1998 ; Petukhova *et al.*, 1999b). More precisely, thanks to its chromatin remodeling activity, RAD54 induces changes in DNA topology, which facilitates accessibility for strand exchange and RAD51-dependent homologous DNA pairing (Petukhova *et al.*, 1999b ; Zhang *et al.*, 2007). Conversely, RAD51 improves ATP hydrolysis and translocation ability of RAD54 on dsDNA (Van Komen *et al.*, 2000 ; Mazina & Mazin, 2004). Biochemical studies have revealed that RAD54 is able to dissociate RAD51 from dsDNA in an ATP-dependent manner (Solinger *et al.*, 2002 ; Li *et al.*, 2007). This action is believed to prevent the nonspecific association of RAD51 with bulk chromatin and to provide DNA polymerase access to the 3'-OH primer terminus to initiate the DNA synthesis reaction in the nascent D-loop (Heyer *et al.*, 2006). In accordance to this, Rad51 foci fail to disappear in yeast *rad54* mutants (Miyazaki *et al.*, 2004). In addition, RAD54 mediates the ATP hydrolysis-driven branch migration of Holliday junctions (Bugreev *et al.*, 2006), which is consistent with its high affinity for branched DNA molecules (Mazina *et al.*, 2007). Finally, RAD54 stimulates resolution of Holliday junctions *via* physical interactions with structure specific resolvase MUS81-EME1 (Mus81-Mms4 in yeast) (Mazina & Mazin, 2008 ; Matulova *et al.*, 2009). RAD54-mediated enhancement of

HJ-specific resolvase function is evolutionarily conserved, although some differences exist. For example, this nuclease enhancement does not require ATP binding nor its hydrolysis by RAD54 in budding yeast (Matulova *et al.*, 2009) while this activity is ATP-dependent in human (Mazina & Mazin, 2008).

Relatively little information is available regarding RAD54 in the plant kingdom. Osakabe et al. (2006) have identified and characterized the RAD54 orthologue in *Arabidopsis thaliana*. AtRAD54 is expressed in all tissues examined and its expression can be induced by γ-irradiation (Osakabe *et al.*, 2006). Arabidopsis *rad54* mutants are viable and fertile, but exhibit increased sensitivity to γ-irradiation and the cross-linking reagent cisplatin (Osakabe *et al.*, 2006). Two-hybrid experiments revealed that AtRAD54 interacts physically with AtRAD51, as well as interactions between alien proteins (i.e. yeast Rad54 with AtRAD51 and yeastRad51 with AtRAD54) (Osakabe *et al.*, 2006 ; Klutstein *et al.*, 2008). In addition, AtRAD54 is able to complement some DNA repair deficiencies of yeast *rad54* mutant cells (Klutstein *et al.*, 2008), suggesting that RAD54 functions are conserved across species. Subsequent analyses in Arabidopsis have shown that AtRAD54 is extremely important in SDSA, but not required in SSA recombination (Roth *et al.*, 2012) and finally, a recent study has demonstrated that AtRAD54 is also involved in geminiviral DNA replication (Kaliappan *et al.*, 2012). Further investigations are needed to specify the AtRAD54 functions at molecular level and to expand these observations to other plants.

A RAD54 paralogue has been identified in budding and fission yeasts and in mammals (**Table 1**), but not in plants and invertebrates to date. RAD54B, the mammal RAD54 paralogue, shares similar biochemical activities with RAD54 (Tanaka *et al.*, 2002 ; Wesoly *et al.*, 2006 ; Zhang *et al.*, 2007). It is also a dsDNA-dependent ATPase that translocates on duplex DNA leading to DNA topological changes and transient opening of the DNA strands. Similar to RAD54, RAD54B interacts with RAD51 (although this interaction may be indirect)

(Tanaka *et al.*, 2000) and promotes RAD51-mediated D-loop formation (Wesoly *et al.*, 2006). Conversely, RAD51 also enhances the activities of RAD54B in human (Wesoly *et al.*, 2006). In addition, RAD54B also stimulates the DNA strand-exchange activity of DMC1, the meiosis-specific recombinase (Sehorn *et al.*, 2004 ; Sarai *et al.*, 2006). In mouse embryonic stem cells, ablation of RAD54 leads to a mild reduction of HR efficiency while the absence of RAD54B has very little effect. However, the absence of both proteins dramatically reduces HR efficiency (Wesoly *et al.*, 2006). The involvement of RAD54B in HR is thus revealed in absence of RAD54. Experiments of MMC sensitivity of single and double mutants indicate that RAD54 and RAD54B function synergistically to protect mice from DNA damages (Wesoly *et al.*, 2006).

The RAD54 homologue identified in *S. cerevisiae* and *S. pombe* is called Rdh54 (Rad homologue 54) or Tid1 (two-hybrid interaction with Dmc1) (Dresser *et al.*, 1997 ; Klein, 1997 ; Catlett & Forsburg, 2003). Despite their biochemical similarities, biological functions of yeast Rad54 and Rdh54 exhibit important differences (**Table 1**). Whereas Rad54 is involved in mitotic DSB repair, Rdh54 appears to act in both mitotic and meiotic recombination. In accordance to this, Rad54 and Rdh54 physically and functionally interact with Rad51 (Jiang *et al.*, 1996 ; Clever *et al.*, 1997 ; Petukhova *et al.*, 2000 ; Mazin *et al.*, 2003 ; Raschle *et al.*, 2004 ; Santa Maria *et al.*, 2013) while only Rdh54 has been found to interact with Dmc1 (Dresser *et al.*, 1997 ; Catlett & Forsburg, 2003). Although its role in meiosis is not fully understood, Rdh54 appears to promote colocalization of Rad51 and Dmc1 (Shinohara *et al.*, 2000) and to control sister chromatid cohesion to facilitate interhomologue recombination and chromosome segregation (Shinohara *et al.*, 2000 ; Shinohara *et al.*, 2003 ; Kateneva *et al.*, 2005). Rdh54 is also implicated in Rad51 and Dmc1 dissociation from dsDNA to avoid recombinase association at nonrecombinogenic sites and to facilitate DNA synthesis initiation in the nascent D-loop structure (Chi *et al.*, 2006a ; Holzen *et al.*, 2006 ;

Shah *et al.*, 2010). Thanks to meiotic functions of Rdh54, the yeast *rad54* mutant only exhibits minor defects in meiotic recombination (Klein, 1997). Moreover, Rdh54 seems to be involved in broader processes: it participates in the coordination of some repair pathways of DNA lesions (Latypov *et al.*, 2010) and may facilitate communication between DNA repair and checkpoint control (Lee *et al.*, 2001). These findings indicate that (1) aside from some functional overlap, yeast Rad54 and Rdh54 have independent functions in HR, DSB repair and other processes and that (2) yeast Rdh54 does not appear to be the functional equivalent to mammal RAD54B, based on mutant phenotypes, particularly the meiotic mutant phenotype.

	RPA	RAD52	RAD59	RAD54	RAD54B
H. sapiens	*RPA = replication protein A, which is comprised of three subunits of 70, 32 and 14kDa [1], was first identified as an indispensable component of simian virus 40 DNA replication [2]* • forms nuclear foci which colocalize with RAD51 foci after γ-irradiation [3, 4], is present during meiosis throughout prophase I until mid-pachytene [5] • interacts with RAD51 [6], [3], with BRCA1 and BRCA2 [7, 8], with XPF–ERCC1 [9] and with WRN and BLM helicases [10-12] • binds to ssDNA with high affinity [2, 13, 14], and in a sequential binding manner with a 5' to 3' polarity [15] • is actived by phosphorylation at the G1- to S-phase transition, and dephosphorylation occurs at mitosis [16] • functions in DNA replication [2, 13, 17, 18], nucleotide excision repair [19] and homologous recombination [20] **Replication**: its phosphorylation is a critical early step for full ATR activation in response to replication stress and subsequent replication checkpoint arrest [21], UV-induced hyperphosphorylation of RPA depends on DNA replication and expression of ATM protein [22] **NER**: plays a role in DNA damage recognition [23, 24] and in recruiting and positioning of XPG and ERCC1-XPF endonucleases to the lesion site for incision reactions [15, 25], promotes junction cutting by XPF-ERCC1 and XPG nucleases of "loop" substrates [25], participates in the gap-filling reaction [26] **BER**: stimulates the long-patch BER [27] **HR**: is required for the recruitment of ATR to sites of DNA damage and for ATR-mediated Chk1 activation [28], modulates RAD51 activity by physical interaction [3, 6], stimulates the strand transfer activity of RAD51 [29], stimulates WRN and BLM helicase-catalyzed DNA unwinding [10-12]	• expression: low in G0/G1, increased in S and maximum in G2/M [30], expression not induced by γ-irradiation [30] • directly interacts with RPA [31] and RAD51 [32] • preferentially binds ssDNA [33, 34] • has strand-annealing activity *in vitro* [35-37] and strand exchange activity [34] • does not show recombination mediator activity in reconstituted biochemical assays [38] but mediates RAD51 function in human cancer cells deficient in BRCA1, PALB2 [39], or BRCA2 [40], mediates second end capture via its strand annealing activity [41], overexpression of RAD52 enhances spontaneous HR but reduces I-SceI-induced HR [42]		• accumulates as foci at sites of DNA damage in the nucleus after IR-treatment [43], focus formation is impaired in *xrcc2, xrcc3, rad51b, rad51c, rad51d* and *brca2* mutant cells but is RAD52-independent [44] • interacts *in vivo* and *in vitro* with RAD51 and this association is induced by IR [45], interacts with and stimulates DNA cleavage activity of MUS81–EME1 [46] • dsDNA-dependent ATPase [47] which translocates along the helix DNA (ATP-dependent manner) [48] • binds ssDNA and dsDNA with similar affinities, but binding to ssDNA does not stimulate its ATP hydrolysis [49], has a higher binding affinity for branched DNA structures [50] • induces a change in the topology of the DNA by binding to dsDNA [51] which facilitates homologous DNA pairing by the RAD51 protein directly [48], is recruited by RAD51-ssDNA filament to the chromatin of the intact chromosome and that it remodels that chromatin to facilitate accessibility for strand exchange [52], binds Holliday junctions and drives their branch migration [53]	*RAD54 homologue, more closely related to ScRdh54/Tid1 than to ScRad54 [54-56]* • forms nuclear foci that colocalize with RAD51, RAD54, and BRCA1 [54, 55] • constitutively interacts with RAD51 but this interaction may be indirect [54], interacts with DMC1 [57, 58] • shares similar biochemical activities with RAD54 [59], is a DNA-binding protein and hydrolyzes ATP in the presence of dsDNA [55] • seems to play an unique role in homologous recombination [56], stimulates the DNA strand-exchange activity of DMC1 by stabilizing the DMC1-ssDNA complex [57, 58]
M. musculus	• forms foci during prophase I [60-62] and colocalizes with RAD51 on newly synapsed axes formed during meiotic prophase [61], accumulates during late leptotene/early zygotene and persists through to pachytene, before dissociating from the chromatin at around the time CO-designated recombination intermediates [63] • *rpa1* heterozygote: accumulation of lymphoid tumours and early embryonic lethality of the litters [64]	• *rad52* mutant is viable [65] • role in HR is uncertain but mutant has decreased gene targeting [65], expression of two splice variants from the RAD52 gene (normally expressed in adult mouse tissues) in tissue culture cells elevates the frequency of recombination that uses a sister chromatid template [66]		• expression elevated in organs of germ cell and lymphoid development, expression correlated with the meiotic phase of spermatogenesis [67], colocalizes with RAD51 foci following IR [51] • adult *rad54* mice: hypersensitive to MMC [68] but resistant to IR [69], *rad54* embryonic stem cells: sensitive to IR, MMC, and MMS, but not to UV light, reduction of gene targeting [70], *rad54 rad54b* double KO: stronger defects on HR [71] • RAD54 affects DNA DSB repair and sister chromatid exchange [72], RAD54 (but not RAD54B) is needed for a normal distribution of RAD51 on meiotic chromosomes [71], plays a minor role in meiotic recombination as well, mutants are fertile [70], role in telomere length maintenance in mammals [73]	• *rad54b* mice viable with no macroscopic abnormalities up to at least 6 months of age [71], *rad54b* cells: modest to no HR defects, but *rad54 rad54b* cells have stronger defects [71] • contributes to repair of IR and MMC-induced DNA damage, involvement of RAD54B in homologous recombination was revealed in absence of RAD54 so functions synergistically with RAD54 to protect mice from the deleterious effects of MMC [71], Rad54B-mediated HR is not essential for somatic hypermutation [74]

Table 1: RPA, RAD52, RAD59, RAD54 and RAD54B proteins in eukaryotes (1/4)

	RPA	RAD52	RAD59	RAD54	RAD54B
C. griseus	• interacts with XPF and this interaction contributes to efficient NER [74]	• expression: low in G0/G1, increased in S and reached a maximum in G2/M and this increase does not require DNA–PK [30]			
G. gallus		• high abundance of the mRNA in testis [75] • *rad52* mutant cells: no hypersensitive to γ-irradiation, MMS, or cisplatin [76, 77], reduction of targeted integration frequency [76], *rad52-xrcc3* double knockdown cells: non viable and exhibit severe HR defects [78] • RAD52 may play a role in meiotic recombination [75]		• *rad54* mutant: X-Ray sensitive, reduced IG gene conversion rate and targeted integration [79], spontaneous chromosome aberrations [80, 81] and cell death [80], RAD54 deficiency is associated with a loss of the S phase dependent radioresistance to killing [77] • RAD54 is not required for IR-induced DNA DSB repair [77]	
D. rerio	*High. similarity to human RPA (identitical, 55%; conservative, 70%) [82]* • is ubiquitously expressed in embryo, liver, ovary, intestine and in cultured fibroblast cell [82] • inhibits annealing of oligonucleotides *in vitro* [83]	• Loss of function of RAD52 impairs the SSA pathway and results in abnormal embryo development [84] • facilitates annealing of oligonucleotides in the presence of RPA *in vitro* [83]		• belongs to SWI2/SNF2P family [58], uses a mechanism analogous to helicases to translocate on dsDNA [85]	
D. melanogaster	• binds ssDNA very tightly [86] • stimulates the extent and processivity of DNA synthesis of the DNA polymerase alpha [86, 87]			**Okra** *drosophila homolog of RAD54 [88]* • *rad54* deficient flies: normal development, but the females are sterile [89] • is involved in the repair of radiation damages and recombination [89], chromatin remodeling and homologous strand pairing [90, 91], required for both mitotic DNA repair and meiotic recombination [88]	
C. elegans	• forms nuclear foci in response to IR, dependent on WRN and MRE11 [92] but independently of ATM [93] • physically interacts with WRN, a member of the RecQ helicase family [94] • binds ssDNA [94] • stimulates WRN helicase activity [94], stimulates both unwinding and endonuclease activities of CeDNA2, an helicase/endonuclease which remove RNA–DNA primers of Okazaki fragments during DNA replication [95]				
A. thaliana	*Arabidopsis has multiple genes for most RPA subunits [96, 97], two RPA complexes depending of RPA-70kDa subunit: RPA70a and RPA70b* • is present during prophase I of meiosis [98] • *rpa70a* mutation: lethal, *rpa70b* mutation: morphologically normal, hypersensitive to UV-B and MMS [99] • complex containing RPA70a is essential for DNA replication, complex containing RPA70b may function mostly in DNA repair [99], is not required for the meiotic DSB repair, but plays an essential role at later stages in the meiotic recombination pathway that is required for the formation of class I COs [98]	**RAD52-1 & RAD52-2** *the 2 genes encode four ORFs through differential splicing* • are localized in all DNA-containing compartments (nucleus, mitochondria, or chloroplast) [104] • *rad52-1* or *rad52-2* mutant: reduced fertility and mild MMC hypersensitivity [104]		• is expressed in all tissues examined, with the highest levels of expression in flower buds, expression induced by γ-irradiation [100] • physically interacts with RAD51 [100] • *rad54* mutant: viable and fertile [100] • predicted to be a DNA-stimulated ATPases (SWI2/SNF2 family) [101] • not required for SSA, but important for SDSA [102], involved in geminiviral DNA replication [103]	

Table 1: RPA, RAD52, RAD59, RAD54 and RAD54B proteins in eukaryotes (2/4)

	RPA	RAD52	RAD59	RAD54	Rdh54 / Tid1
S. cerevisiae	*S. cerevisiae has a single gene for each subunit of RPA* • forms foci in nuclei at meiotic S-phase and in early prophase I which colocalize with Rad51 and Rad52 [105] • is actived by phosphorylation at the G1- to S- phase transition, and dephosphorylation occurs at mitosis [16] • has higher affinity for ssDNA than Rad51 [106, 107] • functions in the unwinding of the SV40 origin of replication [108], its presence on ssDNA prevents Rad51 from binding *in vitro* [106, 107], arrives at DSB sites prior to Rad51 during the presynaptic phase [109] and binds to ssDNA to prevent secondary structure formation that could potentially lead to inhibitory effects during Rad51 nucleoprotein filament formation [106], during the isoenergetic strand exchange phase RPA ensures unidirectional heteroduplex extension by binding to the displaced ssDNA [110, 111], has a crucial role in meiosis [112], is required for the G1/S and intra-S DNA damage checkpoints [113, 114], binds to regulatory elements in DNA repair and DNA metabolism genes so may be involved in the regulation of a number of DNA repair and DNA metabolism genes [115]	• expressed throughout the cell cycle and is induced by DNA-damaging agents and during meiosis [116, 117], is nuclear and forms discrete foci in response to IR and during S phase of unirradiated cells [118] • interacts directly with RPA [119], RAD51 [120] and Rad59 *in vivo* [121, 122] • binds ssDNA [123] and can anneal DNA strands that are either bare or coated with RPA [124-126] • involved in Rad51 recruitment to DNA [127] and Rad51 nucleofilament formation, mediates the displacement of RPA from ssDNA, mediates Rad51 strand invasion by physically associating with the Rad51 protein [120] and allowing for highly efficient removal of RPA [107], stimulates DNA strand exchange by targeting Rad51 protein to a complex of RPA with ssDNA [128], assists in capturing the second DNA end and promotes its annealing to the D-loop [129, 130], promotes post-invasion steps of both crossover and noncrossover pathways of meiotic recombination [131], is required for SSA [132] and BIR [133] and has a critical role in the HDR pathway [36]	*defective for RAD51-independent spontaneous mitotic recombination between inverted repeats [134]* • interacts *in vivo* with Rad52 [121], can self-associate [122] • binds ssDNA and dsDNA with a preference for ssDNA • can anneal complementary ssDNA [121, 135, 136] • *rad59* mutant: modest defects in several mitotic recombination assays and moderate sensitivity to IR [137], *rad59* mutation can be partially complemented by overexpression of RAD52, suggesting that the functions of both proteins overlap [134] • RAD59 is dispensable in RAD51-dependent homologous recombination pathways but plays an important role in the RAD51-independent repair pathways [134], such as SSA [137-139], its requirement in SSA increases as the repeat length decreases [139], can overcome the inhibitory effect of RPA on annealing of oligonucleotides but can not substitute for Rad52 *in vivo*, even in RAD51-independent recombination events such as SSA [121], regulates association of Rad52 with DSB [140]	*Shares 66% similarity and 48% identity with its human homologue [66, 85]* • expression is damage inducible [116], expression levels increase during the late G1 phase [117, 141], RAD54 focus formation is dependent of Rad51, Rad52 and Rad55 [142] • interacts with both free Rad51 and the Rad51 ssDNA nucleoprotein filament *in vivo* [143] and this interaction is functionally important for recombinational DNA damage repair [144-146], interacts with Mus81-Mms4 [147] • binds ssDNA and dsDNA with similar affinities, although binding to ssDNA does not stimulate ATP hydrolysis [46] • motor protein that translocates along dsDNA in an ATP hydrolysis dependent manner [148] • *rad54* mutant has increased rates of spontaneous chromosome loss [149] • stabilizes the Rad51 nucleoprotein filament but its ATPase activity is not required [143], disassembles rad51:dsDNA filaments [150], promotes Rad51-dependent homologous DNA pairing via ATP hydrolysis-driven change in DNA double helix conformation [135], strongly stimulates the Rad51-mediated strand exchange and its ATPase activity is required [48, 135], acts during the synaptic phase of DNA strand exchange and after the formation of presynaptic Rad51 protein-ssDNA filaments [151], has an additional role in the postsynaptic phase of DNA strand exchange by stimulating heteroduplex DNA extension of established joint molecules in Rad51/Rpa-mediated DNA strand exchange [152], cooperates with Mus81-Mms4 nuclease in the resolution of recombination and replication intermediates [153], plays a relatively minor role in meiotic recombination, due to the presence of a meiosis-specific homologue, Rdh54/Tid1 [154], non required for SSA [155]	*Rdh54: Rad homologue 54 [154] / Tid1: two-hybrid interaction with Dmc1 [156], structural and functional relative of Rad54 [157]* • forms foci at DNA damage sites in a Rad51- and Rad52-dependent manner [142] • Rdh54 physically interacts with Rad51 [158, 159] and Dmc1 [156] • has dsDNA-activated ATPase activity which generates unconstrained negative and positive supercoils in DNA [158], translocates on dsDNA in an ATP dependent manner and can disrupt joint molecules [160, 161] • *rdh54/tidl* mutant: retardation of the repair of meiosis-specific DSBs and delayed formation of physical recombinants [162], modest delay in elongation of Zip1 structures and a more pronounced delay in Zip1 disappearance, reduction of crossover interference [163]; *rad54 rdh54* mutant: fails to form viable meiotic progeny and to repair meiotic DSBs [162] • is involved in a minor pathway of mitotic recombination in the absence of Rad54 [162], promotes Rad51-dependent D-loop formation [158], promotes colocalization of Rad51 and Dmc1 during meiotic recombination [157], promotes dissociation of Dmc1 from nonrecombinogenic sites on meiotic chromatin [164], catalyzes Rad51 removal from dsDNA of nondamage-associated foci [165, 166], controls resolution of cohesin-dependent linkages during meiosis [167], is involved in coordination of different DNA repair systems including mutagenic and recombinagenic pathways as well as nucleotide excision repair [168]

Table 1: RPA, RAD52, RAD59, RAD54 and RAD54B proteins in eukaryotes (3/4)

	spRPA = spSSB	Rad22A+ & Rad22B+	RAD59	Rhp54	Rdh54
S. pombe	*spSSB = S. pombe single-stranded DNA-binding protein* [169] *= SpRPA* [170] • has an important role in DNA replication, repair and recombination *in vivo*, is not required for the DNA damage checkpoint but may play an important role in maintaining the signal recognized by the S-phase checkpoint [171]	*ScRad52 homologs* [172, 173] • Rad22A+ is localized to sites of DSBs *in vivo* • Rad22A+ and Rad22B+ physically interact with each other, with Rhp51 and RPA [174] • *rad22A+* mutation: severe sensitivity to X-rays and strong decrease in recombination (13-fold), *rad22B+* mutation: no decrease in HR and no change in radiation sensitivity, *rad22A–rad22B* double mutant: enhancement of radiation sensitivity [173] • protect dsDNA ends from nuclease digestion, is involved in SSA and HR in vegetative cells [173, 175], Rad22B+ has an auxiliary role in the repair of DSBs [173]		• is ubiquitylated and degraded in G1 phase [176] • *rhp54* mutant: spontaneous minichrosome loss, increased sensitivity to X-rays and MMS, severe defects in recombination [177], little or no reduction in meiotic recombination [174, 178] and produces some viable spores (27%) [178] • may be involved in the recombinational repair of UV and X-ray damage and plays a role in the processing of replication-specific lesions [177], acts with Rdh54 to repair meiotic DSBs [179]	*homologous to budding yeast Rdh54/Tid1* [179] • protein present with appropriate timing for a meiosis specific recombination factor [179] • interacts with Dmc1 and Rhp51 [179] • *rdh54* mutant: no effect on DNA damage repair during the haploid vegetative cell cycle, decrease of meiotic recombination and spore viability with a concomitant increase in sister chromatid exchange during meiosis [179] • is important for meiotic recombination [179]

Table 1: RPA, RAD52, RAD59, RAD54 and RAD54B proteins in eukaryotes (4/4)

	RAD51B (REC2 / RAD51L1)	**RAD51D** (RAD51L3)	**XRCC2**	**RAD51C** (RAD51L2)	**XRCC3**
H. sapiens	• expression induced by both ionizing radiation [180] and UV radiation [181] • physically interacts with RAD51C [182] • binds to ssDNA, dsDNA, and 3'-tailed dsDNA *in vitro* [183] • exhibits protein kinase activity *in vitro* and can phosphorylate P53, cyclin E, and CDK2 [184] • inhibition of RAD51B induces G2/M cell cycle arrest [185]	• physically interacts with XRCC2 [186, 187] and RAD51C [187] • binds to ssDNA, 3'-tailed DNA and dsDNA with different affinities (strongly to ssDNA) [186] • has a role in telomere-length regulation [188]	*XRCC2 = X-ray repair cross complementing 2* • physically interacts with RAD51D [186, 187] • does not show significant DNA binding [189] • XRCC2 depletion has no effect on XRCC3 phosphorylation in response to DSB [190] • enhances the ATP-processing activity of RAD51 by facilitating ADP to ATP exchange though reducing the affinity for ADP [189], stimulates RAD51 strand exchange [189]	• accumulates at DNA damage sites concomitantly with the RAD51 recombinase and is retained after RAD51 [191], formation of foci which colocalise with γH2AX [192] • interaction with RAD51 [192], RAD51D [187], RAD51B [182] and XRCC3 [193] • binds to ssDNA, dsDNA, and 3'-tailed dsDNA *in vitro* with different affinities (strongly to ssDNA) [183] • has an apparent strand exchange activity perhaps though destabilizing dsDNA [183] • *rad51c* mutant cells: hypersensitivity to the DNA-cross-linking agent MMC and moderately increased sensitivity to IR (in S and G2/M phases) [194], reduction in HJ resolution activity [195], RAD51C inhibition induced G2/M cell cycle arrest [185], regulation of ubiquitin-mediated degradation of Rad51 [196] • facilitates checkpoint signaling by promoting CHK2 phosphorylation [191], is essential for XRCC3 phosphorylation [190], is required for HJ processing [197]	*XRCC3 = X-ray repair cross complementing 3* • level of XRCC3 protein was sharply reduced in *rad51c* mutant cells [197], is recruited to DSB early and independently of RAD51 [198] • physically interacts with RAD51 [199] and RAD51C [193] • inhibition of XRCC3 elicits checkpoint defects [185] and increases endoreduplication [200] • is critical for RAD51-mediated recovery of collapsed replication forks [190], is required for restart of HU-stalled replication forks [201], participates in the intra-S-phase checkpoint (phosphorylation dependent) and in the G2/M checkpoint (phosphorylation indep.) [190], is required for the production of extrachromosomal telomeric circles in human alternative lengthening of telomere cells [202]
	BC complex	**DX2 complex**		**CX3 complex**	
	• has ssDNA binding activity [203] • is capital for RAD51 focus formation [192], enhances the homologous DNA pairing activity of Rad51 [203], promotes RAD51-catalyzed strand exchange *in vitro* by suppressing the inhibitory effect of RPA [203]	• binds both ssDNA and dsDNA, forms a filamentous complex with ssDNA *in vitro* • has homologous pairing activity *in vitro* [204] • stimulates resolution of synthetic HJs via its interaction with the BLM helicase protein [205]		• interacts with RAD51 *in vitro* [192] • binds DNA templates containing replication forks and HJs and forms a multimeric ring structure whose subunits are arranged into a flat disc around a central channel *in vitro* [206] • acts downstream of RAD51 recruitment [207], catalyzes homologous pairing [204], is involved in HJ processing [197]	
	BCDX2 complex				
	• interacts with RAD51 *in vitro* [192] • has a high affinity for branched DNA [208] and can recognize nicks in DNA [209], binds DNA templates containing replication forks and HJs and forms a multimeric ring structure whose subunits are arranged into a flat disc around a central channel *in vitro* [206] • catalyzes strand-annealing reaction *in vitro* [208] • acts downstream of BRCA2 recruitment but upstream of RAD51 recruitment [207], is responsible for Rad51 recruitment or stabilization at damage sites [207]				

Table 2 : RAD51 paralogues in eukaryotes (1/3)

	RAD51B	RAD51D	XRCC2	RAD51C	XRCC3
M. musculus	• *rad51b* mutation: embryonic lethal, but partially rescued in a p53-/- background [210] • is essential for cell proliferation [210]	• interacts with RAD51C [211] • *rad51d* mutation: embryonic lethal [212] • has DNA binding activity *in vitro* [213] • is involved in resistance to ICL agents (ATPase motif needed) [211]	• *xrcc2* mutation: embryonic lethal [214] • has low DNA binding activity *in vitro* [213]	• is localized to meiotic chromosomes at pachytene/diplotene [215] • *rad51c* mutation: embryonic lethal [216] • has a DNA binding activity *in vitro* [213] • is required for inter-sister chromatid recombinational repair [217]	
				CX3 complex	
				• is associated with HJ • may play an essential role in the resolution of recombination intermediates prior to chromosome segregation [215]	
C. griseus		• *rad51d* mutant cells: broadly deficient in RAD51 focus formation in response to various agents, but this defect is not invariably associated with increased sensitivity [218]	• *irs1* mutant cells (defective in XRCC2): sensitivity to diverse DNA damaging agents such as IR, UV, monofunctional alkylating agents, camptothecin and MMC [219-221] • regulate the balance between short- and long-tract gene conversions between sister chromatids [222]	• contributes to DNA cross-link resistance, sister chromatid cohesion and genomic stabiliy [223], is involved in maintaining centrosome number in mitosis [224]	• *irs1SF* mutant cells (defective in XRCC3): sensitivity to diverse DNA damaging agents such as IR, UV, monofunctional alkylating agents, camptothecin and MMC [219, 221] • is required for assembly of Rad51 complexes *in vivo* [225], promotes homology-directed repair of DNA damage [226], acts in late stages of recombination (in formation and resolution of HR intermediates), controls the fidelity of HR [227], regulates the balance between short- and long-tract gene conversions between sister chromatids [222], modulates replication fork progression on damaged chromosomes [228]
G. gallus	• is invovled in RAD51 and RAD54 foci formation following IR and cisplatin treatments [229], promotes homologous recombinatinal repair [230]	• *rad51d* mutant cells: spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents (MMC and cisplatin), mild sensitivity to gamma rays [231] • is invovled in RAD51 and RAD54 foci formation following IR and CDDP treatments [229]	• *xrcc2* mutant cells: spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents (MMC and cisplatin), mild sensitivity to gamma rays [231]	• *rad51c* mutant cells : spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents (MMC and cisplatin), mild sensitivity to gamma rays [231]	• *xrcc3* mutant cells: spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents (MMC and cisplatin), mild sensitivity to gamma rays [231] • is invovled in RAD51 and RAD54 foci formation following IR and CDDP treatments [229]
A. thaliana	• is expressed widely and at a higher level in the floral buds than in other tissues [232] • *rad51b* mutant : MMC [233] and cisplatin hypersensitive [232], meiotic hyper-recombination phenotype [234] • is involved in somatic HR but non required for RAD51 foci formation [234], is involved in SSA recombination [235]	• is expressed widely, but at very low levels [236] • is involved in somatic HR but non required for RAD51 foci formation [234], is involved in SSA recombination [235], is involved in both transcription and recombination during the defense response [236]	• *xrcc2* mutant: meiotic hyper-recombination phenotype [234] • is involved in somatic HR but non required for RAD51 foci formation [234], is required for SSA recombination [235]	• *rad51c* mutant: meiotic chomosomal fragmentation and sterility, MMC-sensitive [233, 237, 238] • not required for SSA [102]	• *xrcc3* mutant: sterile due to Spo11-dependent meiotic chromosome fragmentation [239, 240] • mitotic hyporec and MMC hypersensitive [239], non required for SSA [102]

Table 2 : RAD51 paralogues in eukaryotes (2/3)

D. melanogaster	**Dm CG2412** *CG2412 appears to be an orthologue of RAD51D (30% identity and 53% similarity with hRAD51D)* [241, 242] • no mutations in CG2412 have been reported [243]	**Dm CG6318** *CG6318 appears to be an orthologue of XRCC2 (24% identity and 41% similarity with the core region of hXRCC2))* [241, 242] • is expressed exclusively in the female germline [244] • no mutations in CG2412 have been reported [243]	**SPN-D** (spindle-D) *SPN-D protein is most similar to **RAD51C*** [241, 245] • is expressed exclusively in the female germline [244] • physically interacts with SPN-B [246] • *spn-D* mutant has defects in meiotic recombination, but not of repair of DSBs in somatic cells [245] • blocks NHEJ during meiosis [246]	**SPN-B** (spindle-B) *SPN-B protein is most similar to **XRCC3*** [241, 245] • *spn-B* mutants: mildly sensitive to IR [244], meiotic defects [245], somatic defects hard to detect, because few eggs from develop into viable progeny [88] • physically interacts with SPN-D [246] • blocks NHEJ during meiosis [246]
C. elegans	**RFS-1** *Appears to be the sole RAD51 paralog in C. elegans, is most similar to **Rad51D*** [247] • interacts with the *C. elegans* homologs of RAD51 and BRCA2 (CeBRC-2) [248, 249] • mutant: sterile, mildly sensitive to IR [250] • binds to and promotes the disassembly of RAD51 from dsDNA, but not ssDNA filaments *in vitro* [251], is required for RAD51 focus formation after ICL treatment but not after IR [247], performs a specialized role in promoting HR-mediated repair at lesions that block replication fork progression [247], is involved in telomere integrity maintenance [250]			
S. cerevisiae		**Rad55** *Rad55 appears to be an orthologue of **XRCC2*** [241] [252] • physically interacts with Rad51 and Rad57 both *in vivo* and *in vitro* [253] [141] • *rad55* mutant: viable [252], DNA damage sensitivity and DSB repair defects, which can be rescued by Rad51 overexpression and gain-of-function Rad51 mutations [254], SSA increased [255] • is phosphorylated in response to genotoxic stress and this phosphorylation event is required for an efficient DNA repair [256], Rad55 phosphorylation is as a sentinel for DNA damage checkpoint activation [257]		**Rad57** *Rad57 would be an orthologue of **XRCC3** according to Lin and coll. (2006)* • physically interacts with Rad55 [253] [141] • *rad57* mutant: viable [252], DNA damage sensitivity and DSB repair defects, which can be rescued by Rad51 overexpression and gain-of-function Rad51 mutations and Srs2 removal [127, 254]
		Rad55-Rad57 complex • Rad55-Rad57 foci formation is Rad51 dependent [258] • forms a stable heterodimeric complex [258], is integrated into Rad51 nucleofilament and form Rad55-57-51 complexes [259], can interact with Srs2 in a 1:1 ratio and can simultaneously bind both Rad51 and Srs2 [259], has higher affinity for Srs2 than for Rad51 [259] • has no recombinase activity but a recombinase mediator activity [258] • is involved in mitotic and meiotic recombination, favorisation of nucleation, elongation and stabilization of Rad51 filament [259], inhibits Srs2 antirecombinase activity [259], stimulates Rad51 in the strand exchange reaction *in vitro* [258], is not required for SSA [155]		
S. pombe		**Rhp55** *Rhp55 = rad homolog S.pombe 55* [260] • no direct interaction with Rhp51 [261], forms an heterodimeric complex with Rhp57 [261] •acts in one DNA repair pathway together with Rhp51 and Rhp54 proteins, but in a different pathway than the putative Rad52p, is involved in GC but not in SSA, is required for full meiotic recombination [260]		**Rhp57** *Rhp57 = rad homolog S.pombe 57, is phylogenetically related to XRCC3* [262] • strongly interacts with Rhp51 [261], is epistatic function with Rhp51 [262], forms an heterodimeric complex with Rhp55 [261] • is involved in GC but not in SSA

Table 2 : RAD51 paralogues in eukaryotes (3/3)

	SHU1	SHU2	CSM2	PSY3	SWSAP1
S. cerevisiae	*Shu1 = suppressor of Sgs1 HU sensitivity 1, was identified by a screen for non-sgs1Δ suppressors of the top3Δ slow-growth phenotype [263], has homology with human **XRCC2**, is a Rad51 paralogue [264]* • physically interacts with Shu2 and Psy3 [265] • epistatic to Mus81 for MMS sensitivity [266] • favours Rad51 focus formation and suppresses Srs2 focus formation [267], promotes both crossover and non-crossover pathways of meiotic recombination, independently of Srs2 [268]	*Shu2 = suppressor of Sgs1 HU sensitivity 2, was identified by a screen for non-sgs1Δ suppressors of the top3Δ slow-growth phenotype [263]* • physically interacts with Shu1 and Psy3 [265] and with Srs2 [269] • is epistatic to Sgs1 and Mus81 for MMS sensitivity [266] • favors Rad51 focus formation [267] and Rad52 focus formation [263]	*Csm2 = chromosome segregation in meiosis, was initially discovered as part of a genome-wide screen for mutations affecting chromosome segregation in meiosis [270]* • shares similar ATP core domain architecture of proteins that belong to the Rad51 family [271] • physically interacts with Psy3 [265], with Rad51 (dependent on Rad55) and with Rad55 (independently of Rad51) [255] • is epistatic to Rad55 [255, 272] • *csm2* mutant: SSA more efficient than in wt [255]	PSY3 = *platinum sensitivity,* was initially identified as part of a genome-wide screen for mutants sensitive to platinum [273], has homology with human **RAD51D**, Rad51 paralogue [264] • shares similar ATP core domain architecture of proteins that belong to the Rad51 family [271] • physically interacts with Csm2, Shu1 and Shu2 [265] • is epistatic to Srs2 [265] and to Rad55 [272] • *psy3* mutant: SSA more efficient than in wt [255] • functions in the same pathway as Mms2 [265]	
			CSM2-PSY3 • is a structural mimic of the Rad51-dimer [274] • preferentially binds synthetic forked DNA or 3'-DNA overhang substrates • recruits the Shu complex to HR substrates, needed for efficient recruitment of Rad55 to DNA repair foci after DNA damage [255], binds and stabilizes the Rad51-ssDNA complex independently of nucleotide cofactor *in vitro* [274]		
	SHU / PCSS complex • Shu1, Shu2, Csm2, Psy3 form a stable complex *in vivo* and *in vitro* [263], form a nucleoprotein filament by binding non specifically to DNA, like Rad51 [275] • the 4 proteins belong to the same epistatic group [263], are epistatic to Rad52 [263] and are implicated in error-free post-replicative repair (members of the Rad6 epistasis group [265]) • Shu complex promotes homologous recombination while suppressing error-prone DNA repair [263, 265, 276], inhibits the Srs2 anti-recombinase [267], is involved in error-free DNA-damage tolerance pathway [265] by recruiting the Rad55- Rad57 complex and the HR machinery to damage sites [272], may have specific function during S phase [265], functions upstream of the Sgs1-Top3-Rim1 pathway [265], acts to promote the formation of recombination structures in replication-associated recombinational repair, independently of Mph1 and Mms2 [277], binds to meiotic DSB sites and is required for formation of Rad51 complexes on meiotic chromosomes, has a critical role in meiotic recombination in mediating assembly of Rad51 nucleoprotein filaments [274]				
S. pombe	**RLP1** *Rlp1 = RecA-like protein [278], is most closely related to **XRCC2** [264]* • interacts with Rhp57 [278] • *rlp1* mutant shows a moderate reduction of mitotic recombination [278], and a decrease of the frequency of intergenic recombination (crossovers), but an increase of GC recombination [279]	**SWS1** *Sws1 = SWIM domain-containing and Srs2-interacting protein 1, is related to S. cerevisiae Shu2 [264]* • physically interacts with Srs2 [264] • controles a very early stage of HR [264]		**RDL1** *Rdl1 = Rad51D-like protein [264], is most closely related to **RAD51D** [264]* • has a more complex set of functions than Rlp1 and Sws1 [264]	
	RPL1-SWS1-RDL1 RPL1, SWS1 and RDL1 form a stable complex [264]				

Table 3: SHU complex proteins in eukaryotes (1/2)

<table>
<tr><td rowspan="3">H. sapiens</td><td rowspan="3"></td><td>SWS1</td><td rowspan="2"></td><td>SWSAP1</td></tr>
<tr><td>• associates with RAD51D and XRCC3 [264]
• sws1 mutant: reduction in number of RAD51 foci [264]
• has pro-recombinogenic activity [264]</td><td>SWSAP1 = SWS associated protein 1, has similarity to archeal RadA [280]
• interacts with RAD51, RAD51B, RAD51C, RAD51D, XRCC3 [280]
• preferentially binds ssDNA [280]
• is a DNA-stimulated ATPase [280]
• swsap1 mutant: reduction in number of Rad51 foci [280]</td></tr>
<tr><td colspan="3">SWS1-SWSAP1</td></tr>
<tr><td colspan="4">• form a stable complex
• is a non-essential HR accessory factor, which affects the efficiency and/or timing of HR [280]</td></tr>
<tr><td rowspan="2">G. gallus</td><td rowspan="2"></td><td>SWS1</td><td rowspan="2"></td></tr>
<tr><td>• sws1 mutant: slight decrease in IR-induced RAD51 focus formation
• its contribution to HR repair is less significant than that of BRCA1, BRCA2 and the RAD51 paralogues [281], requires BRCA2 to contribute to HR [280]</td></tr>
</table>

Table 3: SHU complex proteins in eukaryotes (2/2)

	BRCA1	BRCA2
H. sapiens	*Breast Cancer susceptibility gene 1, tumour suppressor gene* • interacts with RAD51 [282] and with BRCA2 [283] in mitotic and meiotic cells, interacts *in vitro* and *in vivo* with RAD50 [284] • binds strongly to DNA and displays a preference for branched DNA structures [285] • E3-ubiquitin ligase. exhibits a ubiquitin ligase activitiy *in vitro* [286, 287], this activity is required for its tumor suppressor function [129] • has a broader role upstream of BRCA2, participating in various cellular processes in response to DNA damage [288], inhibits the nucleolytic activities of the Mre11-Rad50-Nbs1 complex [285], BRCA1–CtIP complex promotes CtIP-mediated 5′-end resection of DSBs [289], BRCA1–PALB2 interaction is a prerequisite for the recruitment of BRCA2 and RAD51 to the site of DNA damage and for homologous recombination [290-292], is involved directly or indirectly in the repair of crosslinked DNA [293], is involved, in complex with BARD1, in G1/S, S-phase and G2/M checkpoints activation [294] [295], ubiquitinates G2/M cell cycle proteins, cyclin B and Cdc25C, leading to their accelerated degradation which permits mitotic entry [296], facilitates the ability of ATM and ATR to phosphorylate downstream substrates that directly influence cell cycle checkpoint arrest and apoptosis [297], may regulate telomere length and stability [298]	*Breast cancer susceptibility gene 2, tumour suppressor gene* • forms nuclear foci which colocalize with RAD51 foci after IR treatment of somatic cells [188] • interacts *in vivo* with RAD51 [299] [283], one BRCA2 molecule can bind 6 molecules of RAD51 [300], interacts with DMC1 [301], associates with BRCA1 through PALB2 [290-292], physically interacts with FANCD2 *in vivo* [302] • binds ssDNA and dsDNA [303] • *brca2* mutation: impairs HR-mediated DSBR but maintains normal NHEJ [304] • BRCA2 mediates RAD51 filament formation and strand exchange after DNA damage by promoting the assembly of RAD51 onto RPA-coated ssDNA [37, 300, 305, 306], prevents RAD51 from binding to dsDNA }[305], is essential for RAD51 foci formation after DNA damage [307, 308], BRCA2-RAD51 interaction is critical for cellular response to DNA damage caused by MMS [309], is a key regulator of the extension step after strand invasion at replication-dependent DSBs allowing Polη localization at collapsed replication forks and Polh-dependent DNA synthesis [310]
M. musculus	• Most homozygous *brca1* mutations are embryonically lethal [311-313], phenotype of *brca1* mouse embryos mimics that of *rad51* mice, *brca1* cells: telomere dysfunction, chromosome translocations, chromatid aberrations [314-316] gene targeting defects and increase of non homologous integration, sensitive to MMC [317, 318] • is required for RAD51 nuclear focus formation [319], promotes HDR and SSA pathways [317, 320]	• is highly expressed during spermatogenesis and detected in early meiotic prophase I [321, 322] • most homozygous *brca2* mutations are embryonically lethal [312, 323, 324], *brca2* cells show chromatid breaks and aberrant chromatid exchanges [325], expression of a truncated BRCA2 showed a failure in spermatogenesis [321] • promotes HDR and suppresses SSA [320], seems to be required for the correct localization of RAD51 and DMC1 recombinases to DSBs [326]
C.griseus		• physically interacts with FANCD2 *in vivo* [302] • protects the nascent strand from degradation at stalled replication forks [327]
G. gallus	• BRCA1-CtIP complex promotes CtIP-mediated 5′-end resection of DSBs [289], BRCA1 is required for repair of DSBs by homologous recombination but not MMEJ [289]	*Has 37% amino acid identity overall with human BRCA2 [328]* • *brca2* cells: lethal [329], BRCA2 +/- mutation: reduced growth rate, increased cell death, heightened sensitivity to specific DNA damaging agents and reduced RAD51 focus formation after irradiation [329]
D. rerio	[330]	**Fancd1** • expressed in proliferating somatic cells and in meiotic oocytes and spermatocytes, Brca2 transcript is asymmetrically localized to the animal pole of the cytoplasm in developing wild-type oocytes [331] • *brca2* mutations: genome instability, slow growth of tissue culture cells, female-to-male sex reversal, testicular neoplasias [331] and male infertility with meiotic arrest in spermatocytes [332] • acts in establishing or maintaining the architecture of the vertebrate oocyte nucleus [331], is involved in ovarian development and tumorigenesis in reproductive tissues [332]
D.melano	[333]	• forms nuclear foci after DNA damage [334] • *brca2* null mutant: viable [335] • physically interacts with SpnA (dmRAD51) [334] • required for mitotic and meiotic DNA repair and efficient activation of the meiotic recombination checkpoint [335]

Table 4: BRCA1 and BRCA2 proteins in eukaryotes (1/2)

	BRC-1	BRC-2
C. elegans	*Protein sequence 24% identical and 52% similar to human BRCA1 (delta-exon 11 splice variant)* [336] • *brc-1* animals: high incidence of males (result of X chromosome nondisjunction), elevated levels of p53-dependent germ cell death before and after irradiation, and impaired progeny survival and chromosome fragmentation after irradiation [336]	• Brc-2 is a little over a tenth the size of its human counterpart [337], expression higher in oocytes than in other germline cells, and barely detectable in mitotic cells [338], forms foci in response to DNA damage, independently of RAD51 [249] • *brc-2* mutant: embryonc lethal (Martin, 2005), *brc-2* cells: fail to repair meiotic or radiation-induced DSBs by HR [249] • interacts directly with RAD51 *in vivo* and *in vitro* [249] • binds preferentially to ssDNA [249] • stabilized of RAD51-DNA filaments [339], stimulates RAD51 mediated D-loop formation and promotes DNA single-strand annealing [340], plays an essential role in chromosome integrity of germ cells [338], is crucial in meiotic progression [338]
A. thaliana	• is expressed ubiquitously in plant tissues, at levels depending on organ type, with highest levels in flower buds and exponentially growing cell cultures [341], expression is induced by γ-irradiation [341, 342] • *brca1* mutant: fertile, no defect in development, HR defects [342] and mild hypersensitivity to MMC [343] • epistatic to BARD1 in DNA crosslink repair [343], non essential for meiosis [343]	**BRCA2A** = BRCA2(IV) **et BRCA2B** = BRCA2(V) *Arabidopsis has two homologs of the BRCA2 gene, 96.8% identity* [344] • expressed in flower buds, shoot and root apexes and, in the case of BRCA2A, at moderate levels in leaves [344, 345], expression not induced by γ-irradiation [344] • BRCA2 proteins interact with RAD51 and DMC1 [344, 346], existence of the BRCA2A-RAD51 complex *in planta* [345] • *brca2a and brca2b* mutant plants have mild hypersensitivity to genotoxic stresses [347], *brca2a/brca2b* double mutant shows an additive increase in sensitivity to genotoxic stresses, and altered cell cycle progression of shoot apical meristems [347] • essential role at meiosis in mediating the function of both RAD51 and DMC1 [344, 348], important for both somatic and meiotic homologous recombination [348], function in cell proliferation and cell cycle regulation [347], BRCA2A, but not BRCA2B is required for salicylic acid-induced expression of pathogenesis-related proteins [345]
S. cerevisiae		
S. pombe		

Table 4: BRCA1 and BRCA2 proteins in eukaryotes (2/2)

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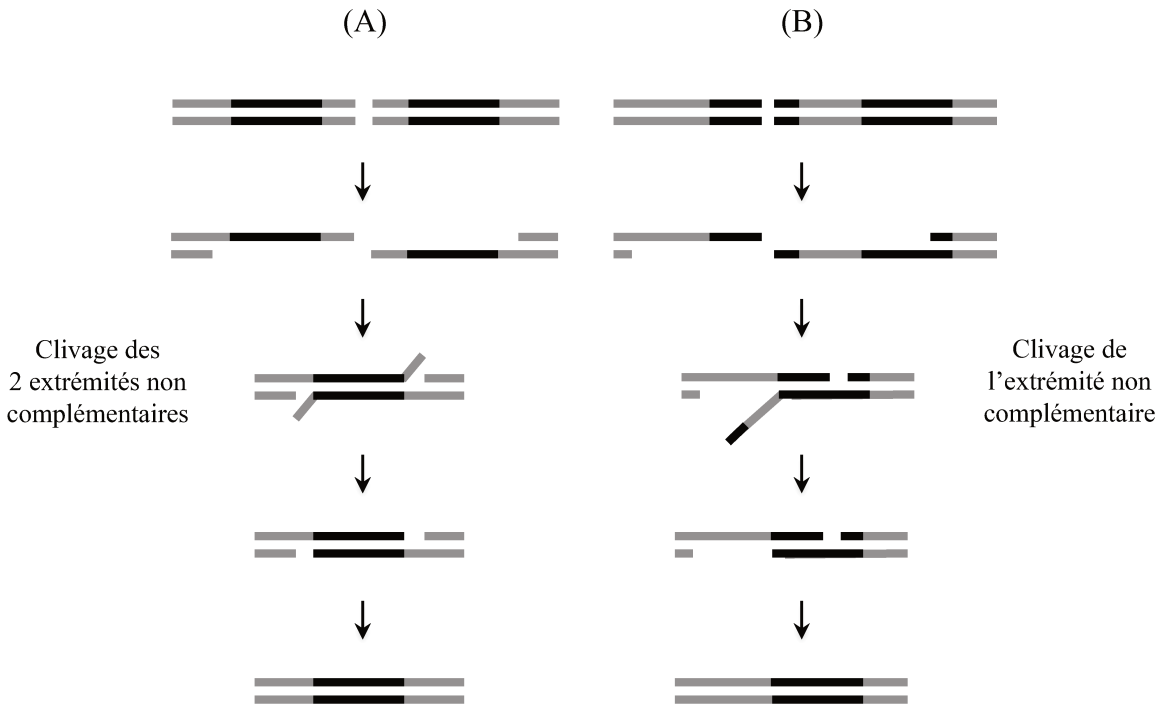


Figure 13 : La réparation d'une CDB par SSA implique l'élimination de deux (A) ou d'une (B) extrémité(s) non complémentaire(s) selon la localisation de la cassure.

(A) Quand la CDB se produit entre deux régions homologues (traits noirs), leur appariement génère deux extrémités d'ADN simple brin 3' sortantes qui devront être clivées pour restaurer l'intégrité de la molécule d'ADN.

(B) Quand la CDB se produit dans l'une des répétitions, une seule extrémité d'ADN doit être éliminée pour amorcer la synthèse d'ADN nécessaire à la réparation.

II.3. Les protéines impliquées dans l'élimination des extrémités non complémentaires

Au cours de la voie d'hybridation simple brin (SSA), les deux régions homologues situées de part et d'autre de la CDB s'apparient grâce à l'activité de RAD52 (et Rad59 chez la levure). Cependant, les extrémités d'ADNsb 3' sortantes ne peuvent s'apparier puisqu'elles ne sont pas complémentaires des nouvelles régions flanquantes (**Figure 13 A**). Ces extrémités doivent donc être clivées pour permettre la réparation de chacun des brins. Dans le cas où la CDB se produit dans une des séquences répétées, la réparation par SSA n'implique le clivage que d'une seule extrémité 3' (**Figure 13 B**).

La recombinaison homologue de type conversion génique (DSBR, SDSA, BIR) peut également nécessiter l'élimination d'une ou plusieurs extrémités 3' non complémentaires. Dans la voie SDSA, voie privilégiée pour la réparation des CDB somatiques (Ira *et al.*, 2006), le clivage de ces extrémités est un prérequis à l'initiation de la synthèse d'ADN à deux étapes distinctes : lors de l'invasion du brin dans la double hélice d'ADN non endommagée et lors de l'appariement du brin envahisseur à l'autre extrémité de la cassure (**Figure 14**). Il a été montré que la présence de séquences non homologues au niveau d'une ou des deux extrémités de la CDB n'affecte pas le choix de la voie de réparation et la proportion des produits de CO et de NCO (Ira *et al.*, 2006).

La majorité des études portant sur l'identification et la caractérisation des protéines impliquées dans l'élimination des extrémités non complémentaires ont été conduites chez la levure. Le clivage de ce type d'extrémités est assuré chez *S. cerevisiae* par le complexe Rad1-Rad10 (Fishman-Lobell & Haber, 1992 ; Bardwell *et al.*, 1994 ; Ivanov & Haber, 1995). Il reconnaît les transitions entre ADNsb et ADNdb et clive spécifiquement le brin contenant l'extrémité d'ADNsb 3' sortante (Bardwell *et al.*, 1994). Ce complexe a initialement été identifié comme un acteur du système de réparation par excision de nucléotide (pour revue,

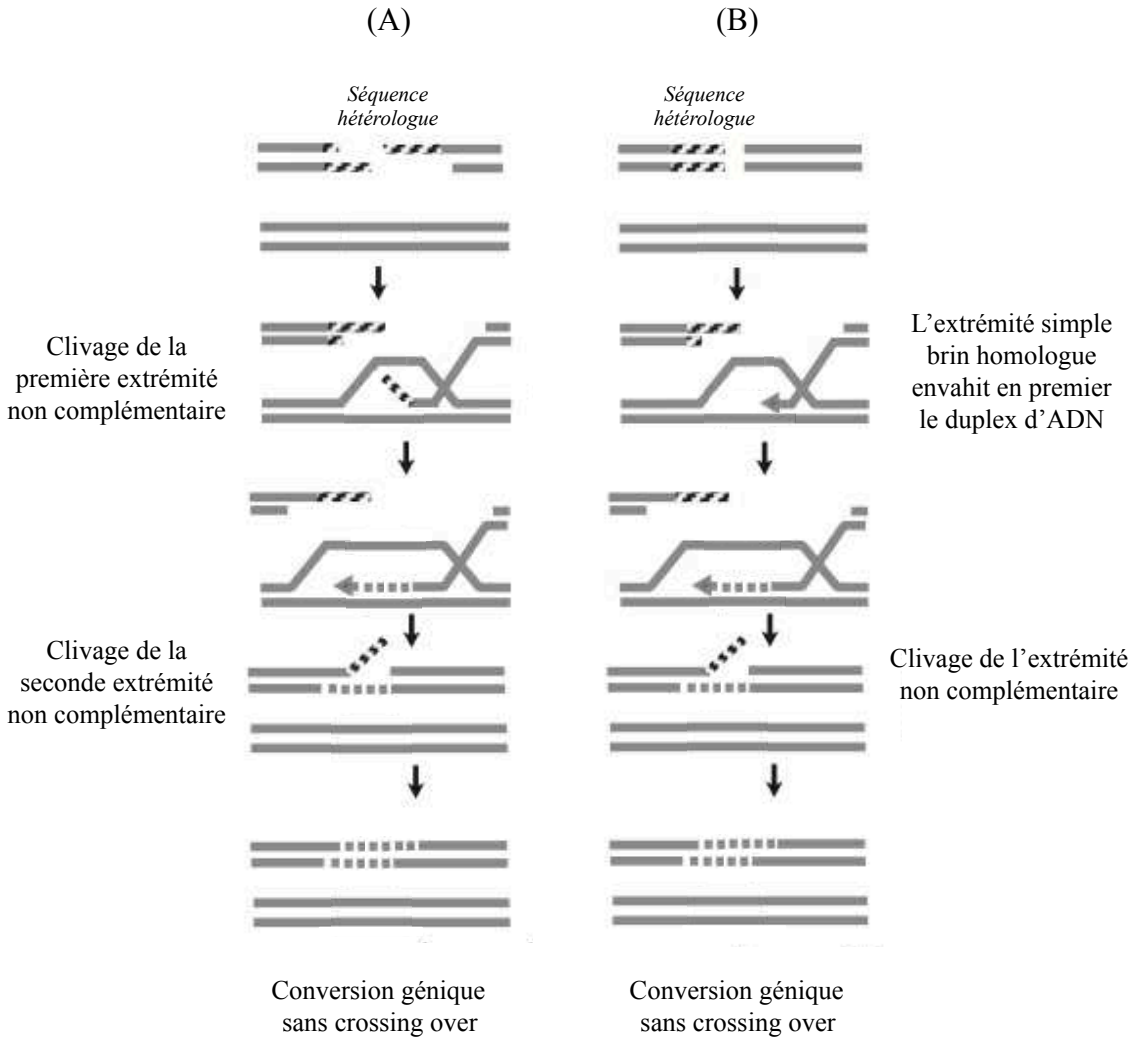


Figure 14 : La réparation par SDSA d'une CDB située dans une séquence hétérologue implique l'élimination de deux (A) ou d'une (B) extrémité(s) non homologue(s) (d'après Ira *et al.*, 2006)

(A) Si une CDB se forme dans une séquence hétérologue (ex : site de restriction de l'enzyme I-SceI), les deux extrémités de la cassure contiennent des séquences non homologues au locus donneur (régions hachurées). L'extrémité 3' non complémentaire du brin réalisant l'invasion doit être éliminée pour initier la synthèse d'ADN utilisant comme matrice la séquence donneuse. Le brin nouvellement synthétisé peut ensuite s'apparier avec l'autre extrémité de la cassure. La non complémentarité des séquences du brin néosynthétisé et du brin d'origine nécessite l'élimination de l'extrémité d'ADNsb 3' sortante.

(B) Si une CDB se forme à une extrémité d'une séquence hétérologue, seule une extrémité de la CDB contient une séquence non homologue au locus donneur (région hachurée). Dans ce cas, c'est principalement l'extrémité homologue qui réalise l'invasion du duplex homologue et initie la synthèse d'ADN. La suite du processus se déroule comme en (A).

voir Prakash and Prakash, 2000). Des analyses ultérieures ont montré que le complexe Rad1-Rad10 est nécessaire au clivage des séquences non homologues au cours du SSA et de la conversion génique (CG) (Fishman-Lobell & Haber, 1992 ; Ivanov & Haber, 1995). Les autres protéines du NER ne sont en revanche pas impliquées dans ce processus (Ivanov & Haber, 1995).

Les protéines Msh2 et Msh3 sont de plus requises à l'élimination des extrémités non homologues dépendante de Rad1-Rad10 (Sugawara *et al.*, 1997 ; Studamire *et al.*, 1999 ; Langston & Symington, 2005 ; Surtees & Alani, 2006). Ces deux protéines forment un complexe de reconnaissance des mésappariements d'ADN impliqué dans le système de réparation des mésappariements. Msh2-Msh3 reconnaît également des structures d'ADN branchées avec une extrémité 3' libre (Sugawara *et al.*, 1997) et se lie rapidement et spécifiquement à la jonction entre ADNsb et ADNdb (Evans *et al.*, 2000 ; Surtees & Alani, 2006 ; Lyndaker *et al.*, 2008). Cette liaison modifie la conformation du complexe à l'origine d'une légère ouverture de la jonction d'ADN (Surtees & Alani, 2006). Grâce à la stabilisation de ces jonctions, Msh2-Msh3 facilite l'élimination des extrémités non homologues dépendante de Rad1-Rad10 au cours du SSA et de la CG (Sugawara *et al.*, 1997 ; Studamire *et al.*, 1999). Aucun autre facteur du système de réparation des mésappariements n'est requis dans ce processus (Saparbaev *et al.*, 1996 ; Sugawara *et al.*, 1997 ; Langston & Symington, 2005).

Des travaux plus récents ont identifié deux nouveaux acteurs de l'élimination de ce type d'extrémités chez la levure : Slx4 *(Synthetic lethal of unknown function protein 4)* et Saw1 *(Single-strand annealing weakened 1)* (Flott *et al.*, 2007 ; Li *et al.*, 2008). Slx4 est une sous-unité du complexe Slx1-Slx4, initialement identifié comme indispensable à la survie cellulaire en absence de l'hélicase Sgs1 (Mullen *et al.*, 2001). Slx4 est phosphorylé en réponse aux dommages de l'ADN grâce aux kinases Mec1 et Tel1 (Flott & Rouse, 2005 ; Flott *et al.*, 2007). Cette activation de Slx4 joue un rôle essentiel dans la réparation par SSA mais le

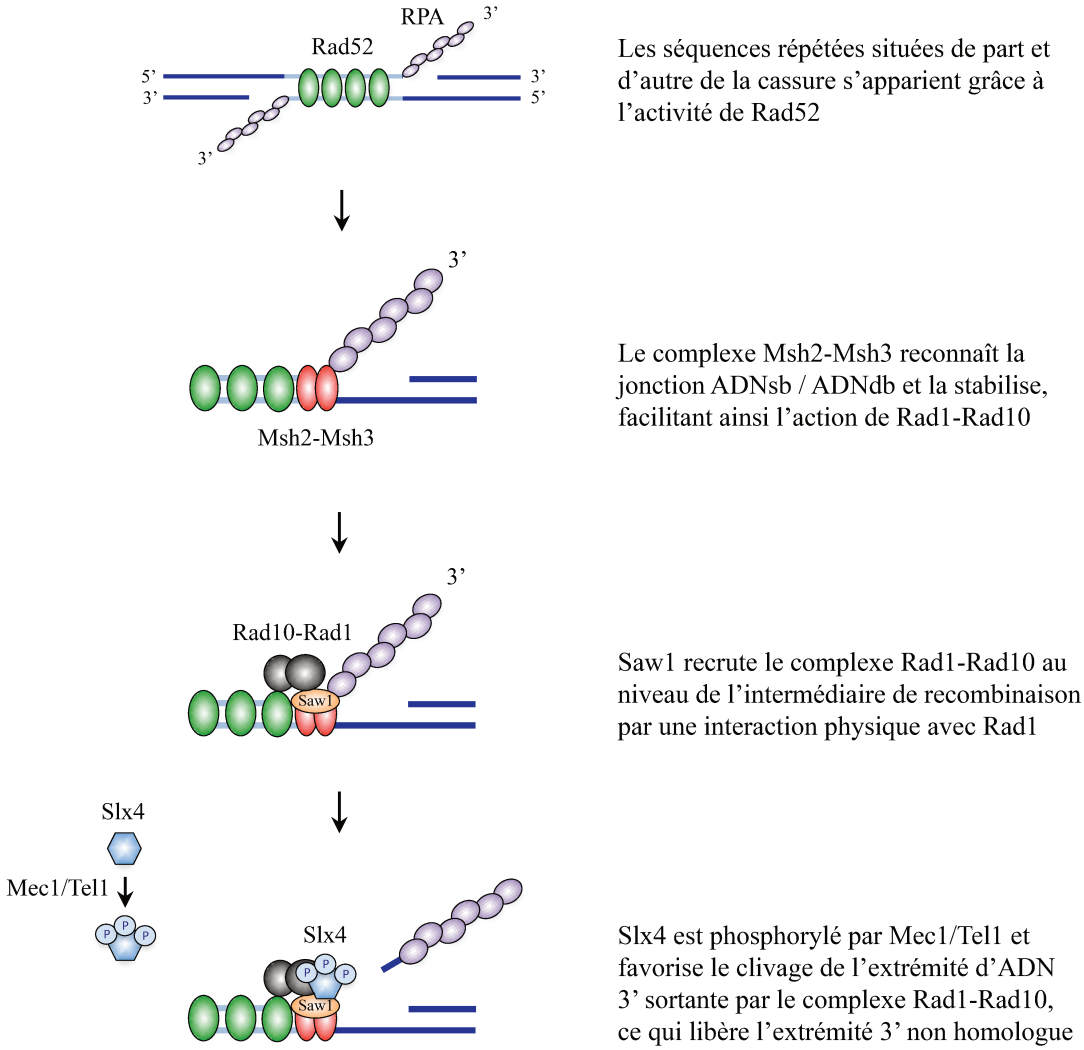
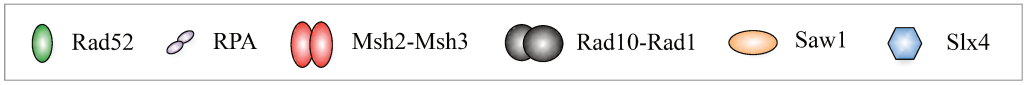


Figure 15 : Modèle d'élimination des longues extrémités 3' non homologues au cours du SSA chez *Sacharomyces cerevisiae* (d'après Lyndaker *et al.*, 2009 et Li *et al.*, 2013)



mécanisme précis n'est pas connu (Toh *et al.*, 2010). Saw1 interagit physiquement avec plusieurs acteurs du SSA : Rad1-Rad10, Msh2-Msh3 et Rad52 (Li *et al.*, 2008). La délétion de Saw1 ou l'expression d'une protéine Saw1 mutée dans son domaine d'interaction avec Rad1-Rad10 abolit la liaison de Rad1-Rad10 aux extrémités d'ADNsb 3' sortantes et bloque le SSA (Li *et al.*, 2008). Une étude récente a de plus montré que Saw1 est une protéine de liaison à l'ADN qui se lie spécifiquement et avec une haute affinité aux structures d'ADN branchées et facilite le recrutement de Rad1 au niveau des extrémités 3' *in vivo* et *in vitro* (Li *et al.*, 2013). Saw1 favorise également l'activité de clivage du complexe Rad1-Rad10 *in vitro* (Li *et al.*, 2013). L'ensemble de ces données indique que Saw1 joue (au minimum) un rôle essentiel dans le recrutement de Rad1-Rad10 au niveau des intermédiaires de recombinaison présentant une extrémité d'ADNsb 3' sortante. Le modèle actuel d'élimination des longues extrémités 3' non homologues au cours du SSA est présenté **figure 15**.

Cependant, l'implication de Rad1, Rad10, Msh2, Msh3 et Saw1 dans le SSA et la CG dépend du nombre et de la longueur des extrémités non homologues (Pâques & Haber, 1997 ; Colaiácovo *et al.*, 1999). En effet, quand ces extrémités ont une longueur inférieure à 30 nucléotides, la CG reste efficace en absence de Rad1, Msh2 ou Msh3 (Pâques & Haber, 1997). Ces protéines sont donc essentielles à la CG uniquement quand les deux extrémités de la CDB contiennent des séquences non homologues de plus de 30 nucléotides. En accord avec ces résultats, il a été montré récemment que l'affinité de Saw1 pour les structures d'ADN branchées dépend de la longueur de l'extrémité 3' sortante : Saw1 ne se lie pas aux extrémités dont la taille est inférieure à 10 nucléotides, puis l'affinité de la protéine augmente avec la longueur de l'extrémité (Li *et al.*, 2013). Les extrémités courtes sont prises en charge par un mécanisme indépendant de Rad1-Rad10 et Msh2-Msh3 qui dépend de l'activité de relecture 3'-5' de la polymérase δ (Pâques & Haber, 1997). De plus, Colaiacovo *et al*. ont montré que dans les mutants *rad1* ou *msh2*, quand les deux extrémités de la CDB présentent de longues

séquences non homologues, l'efficacité de la CG est réduite d'un facteur 90 comparé à une CDB sans extrémité non homologue. En revanche, quand une seule longue extrémité est présente, l'efficacité de la réparation n'est réduite que d'un facteur 5 (Colaiácovo *et al.*, 1999). Il existe donc chez la levure une voie alternative d'élimination des extrémités non homologues mais celle-ci est moins efficace que le mécanisme dépendant de Rad1-Rad10. Msh2 inhiberait cette voie alternative (Manthey *et al.*, 2009). L'hypothèse proposée pour expliquer que Rad1-Rad10 est indispensable seulement quand deux extrémités non homologues sont présentes est la suivante. Lors de l'invasion initiale de l'ADNsb dans le duplexe homologue, la présence d'une extrémité non complémentaire rend la jonction particulièrement instable et serait un bon substrat pour Rad1-Rad10 (Sugawara *et al.*, 1997). Si une seule extrémité de CDB contient une séquence non homologue, c'est l'autre extrémité qui assurera l'invasion de brin (**Figure 14 A**). La jonction formée ensuite au niveau de l'extrémité non invasive serait une structure plus stable (puisqu'une partie de la molécule est correctement appariée), un substrat moins efficace pour Rad1-Rad10.

Chez les Mammifères, XPF et ERCC1 (les homologues de Rad1 et Rad10, respectivement) forment également un hétérodimère capable de cliver spécifiquement les extrémités d'ADNsb 3' sortantes à la jonction entre ADNsb et ADNdb (Biggerstaff et al., 1993). Alors que ERCC1 est catalytiquement inactif, XPF fournit l'activité endonucléasique structure spécifique du complexe. Chacun d'eux est instable en absence de son partenaire (Gaillard & Wood, 2001). En plus de son rôle essentiel dans le NER, il a été démontré que le complexe XPF-ERCC1 est requis dans les voies du SSA et de la CG dans les cellules de Mammifères (Adair, 2000 ; Sargent *et al.*, 2000 ; Al-Minawi *et al.*, 2008). Ce rôle est cependant dépendant de la longueur des extrémités non homologues : XPF-ERCC1 est indispensable au clivage des longues extrémités (> 270 nucléotides), alors que les extrémités courtes (11 et 18 nucléotides) sont efficacement éliminées en absence de ERCC1 (Adair, 2000 ; Sargent *et al.*, 2000). Ces résultats mettent donc en évidence une excellente conservation des

fonctions de ce complexe entre la levure et les Mammifères. Motycka *et al.* ont de plus démontré que la protéine XPF humaine interagit directement avec la protéine RAD52. Cette interaction stimule d'une part l'activité endonucléasique de XPF-ERCC1 et atténue d'autre part l'activité d'hybridation de brins de RAD52 (Motycka *et al.*, 2004), ce qui favorise par conséquent l'élimination des extrémités 3' des intermédiaires de recombinaison.

Chez *Arabidopsis*, le rôle de AtXPF et AtERCC1 dans l'élimination de longues extrémités non homologues (> 230 nucléotides) au cours du SSA a été vérifié (Dubest *et al.*, 2002 ; Dubest *et al.*, 2004). Dubest *et al.* (2004) ont également montré que AtERCC1 est impliqué dans la réparation de substrats de recombinaison présentant des séquences répétées en orientation inverse. L'utilisation de ce type de cassettes permet de quantifier les évènements de CG chromosomiques. La position de la CDB dans le substrat étant aléatoire, sa réparation implique dans la majorité des cas l'élimination de longues extrémités non homologues. Le complexe AtXPF-AtERCC1 est donc également requis au clivage des longues extrémités non complémentaires au cours de la CG chez les plantes (Dubest *et al.*, 2004).

II.4. Les protéines impliquées dans la dissociation des intermédiaires de recombinaison

Au cours des voies de RH dépendantes de RAD51, l'invasion d'une région homologue par une extrémité d'ADNsb 3' sortante aboutit à formation de la boucle D. Suite à la synthèse d'ADN initiée à partir de l'extrémité 3', les deux molécules jointes doivent être dissociées pour achever le processus. Dans le cas d'une réparation par SDSA, le brin nouvellement synthétisé est dissocié du duplexe d'ADN par l'action d'hélicases, telles que Srs2, Sgs1 et Mph1 chez *S.cerevisiae* (Ira *et al.*, 2003 ; Dupaigne *et al.*, 2008 ; Muira *et al.*, 2012 ; Mitchel *et al.*, 2013). La situation est plus complexe dans le cas d'une réparation de type DSBR puisque la capture de l'extrémité non invasive (par une liaison covalente avec le brin néosynthétisé)

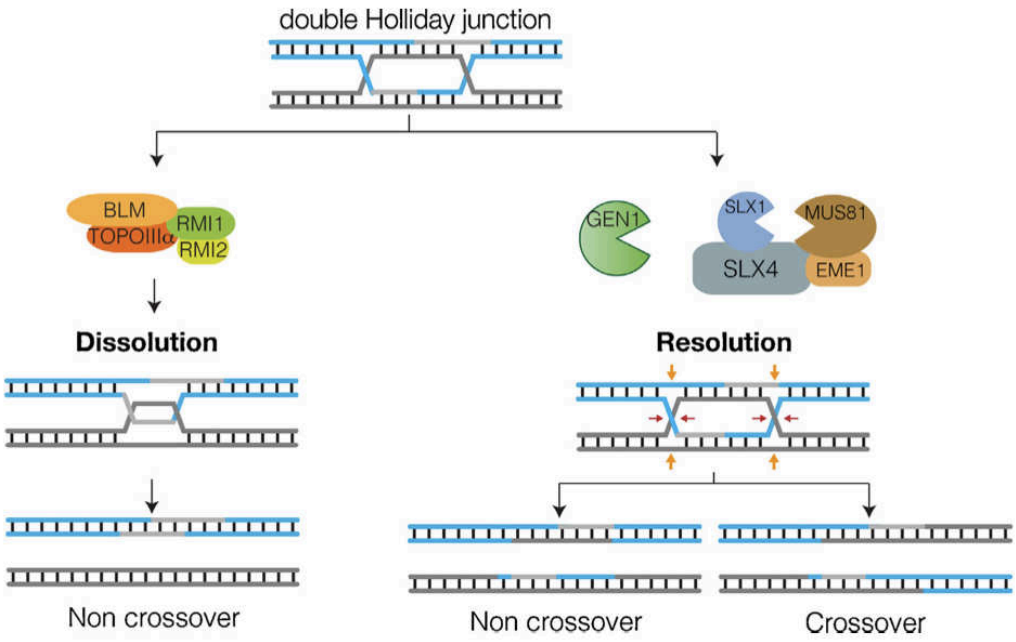


Figure 16 : Voies de dissolution et de résolution des doubles jonctions de Holliday chez les Mammifères (d'après Matos *et al.*, 2014)

Le complexe BLM-TOTOIIIα-RMI1-RMI2 désengage la double JH par un mécanisme de « dissolution » et génère exclusivement des produits de non crossing-over. MUS81-EME1 et SLX1-SLX4 interagissent pour former un complexe qui résout les JH par clivage endo-nucléasique et peut générer des produits de crossing-over ou non crossing-over selon l'orientation des clivages. GEN1 constitue une voie parallèle de résolution des JH.

conduit à la formation d'une double jonction de Holliday (JH) (Szostak *et al.*, 1983) (**Figure 8**). La séparation des deux molécules d'ADN implique soit la dissolution, soit la résolution de cette double jonction, chacun de ces processus nécessitant l'action de protéines différentes.

II.4.1. Les protéines de la dissolution des jonctions de Holliday

La dissolution des dJH est assurée par l'action combinée d'une hélicase, qui permet le rapprochement des JH par migration des branches, et d'une topoisomérase, indispensable au relâchement des surenroulements générés. La rencontre des deux JH forme une structure hémicaténaire, qui sera prise en charge par la topoisomérase pour séparer les deux doubles hélices d'ADN (**Figure 16**). Chez *S. cerevisiae*, la dissolution des JH est assurée par l'hélicase Sgs1 associée en complexe avec la topoisomérase Top3 et la protéine Rmi1 *(RecQ-mediated genome instability)* (Chang *et al.*, 2005 ; Mullen *et al.*, 2005). Rmi1, grâce à sa haute affinité pour les structures d'ADN cruciformes, permettrait le recrutement de Sgs1-Top3 au niveau des JH (Mullen *et al.*, 2005 ; Cejka *et al.*, 2010b).

Chez les Mammifères comme chez les plantes, des homologues des différentes protéines de levure ont été identifiés. La protéine BLM *(Bloom syndrom protein)* humaine et la protéine AtRECQ4A d'*Arabidopsis* sont les homologues de Sgs1 ; TOPOIIIα et AtTOP3a, les homologue de Top3 et enfin RMI1, RMI2 et AtRMI1, les homologues de Rmi1. Les fonctions de chacune de ces protéines sont extrêmement bien conservées au sein des Eucaryotes (Hartung *et al.*, 2008 ; Bernstein *et al.*, 2010).

Dans les cellules mitotiques, la dissolution des JH semble être la voie privilégiée de réparation des intermédiaires de recombinaison (pour revue, voir Heyer *et al.*, 2010). La réaction de dissolution des JH, qui aboutit exclusivement à des produits de NCO, est essentielle pour éviter les CO, comme le montre l'augmentation de la fréquence des CO dans les cellules déficientes pour BLM (Wu & Hickson, 2003).

II.4.2. Les protéines de la résolution des jonctions de Holliday

Une activité résolvase des JH a pour la première fois été observée dans des extraits préparés à partir de tissus de thymus de veau (Elborough & West, 1990) puis à partir de cellules en culture (Hyde *et al.*, 1994 ; Constantinou *et al.*, 2001). L'existence de plusieurs résolvases chez les Eucaryotes a compliqué la recherche de simples mutants déficients pour la résolution des JH. D'abord, l'hétérodimère Mus81-Mms4 (MUS81-EME1 chez les Mammifères) a été identifié chez la levure *S. pombe* (Boddy *et al.*, 2001) et chez l'Homme (Chen *et al.*, 2001) comme une endonucléase capable de cliver les JH, ainsi qu'une large gamme de structures branchées. La protéine MUS81 (une protéine homologue de XPF) possède l'activité endonucléasique du complexe, EME1 est en revanche une sous-unité non catalytique. Cependant, la capacité du complexe à cliver les JH intactes est très limitée par rapport aux autres types de structures, ce qui suggère que Mus81-Mms4 pourrait prendre en charge des JH déjà maturées (dont un brin est déjà clivé par exemple) (Bastin-Shanower *et al.*, 2003 ; Ciccia *et al.*, 2003).

Un deuxième hétérodimère à activité endonucléasique structure spécifique a été identifié chez plusieurs organismes dont la levure et l'Homme, il s'agit de SLX1-SLX4. Ce complexe est également capable de cliver les JH (Fekairi *et al.*, 2009 ; Muñoz *et al.*, 2009 ; Svendsen *et al.*, 2009). SLX1 possède le domaine endonucléase alors que SLX4 réalise les interactions avec de multiples nucléases impliquées dans le clivage (symétrique ou asymétrique) des JH (pour revue, voir Svendsen & Harper, 2010). Il a été montré récemment que SLX1-SLX4 interagit directement avec MUS81-EME1 et que ce complexe multi-protéique agit de manière plus efficace que les hétérodimères individuels (Wyatt *et al.*, 2013). L'augmentation de la résolution des JH requière l'action coordonnée des activités nucléases de MUS81 et SLX1 (Wyatt *et al.*, 2013).

Des travaux récents, menés au sein du laboratoire de Stephen West, ont permis l'identification de la protéine humaine GEN1 - une nouvelle résolvase des JH - et son orthologue Yen1 chez la levure (Ip *et al.*, 2008 ; Rass *et al.*, 2010). GEN1 lie spécifiquement les JH et permet leur résolution par un mécanisme de double incision dans lequel chacun des brins d'ADN de la jonction est clivé symétriquement et simultanément (Rass *et al.*, 2010). Bien que GEN1 soit une endonucléase monomérique, deux monomères GEN1 sont capables de s'associer au niveau de la JH. Cette dimérisation est nécessaire à la résolution efficace des JH, puisqu'elle fournit les deux sites actifs requis au double clivage (Rass *et al.*, 2010). Des analyses d'épistasie ont révélé que GEN1 agit dans une voie de résolution des JH distincte de la voie dépendante de MUS81-EME1 et SLX1-SLX4 dans les cellules humaines (Wyatt *et al.*, 2013). Il existe donc chez les Mammifères comme chez la levure, deux voies de résolution des JH (**Figure 16**). Selon l'orientation des clivages, ces voies peuvent aboutir à des produits de CO ou de NCO (**Figures 8 et 16**).

Les mécanismes de résolution des JH chez les plantes n'ont pas encore été élucidés. Un orthologue de MUS81 a été identifié chez Arabidopsis (AtMUS81) ainsi que deux orthologues de EME1 (AtEME1a et b). Des analyses biochimiques ont montré que les deux complexes AtMUS81-AtEME1a et AtMUS81-AtEME1b sont capables de cliver les JH (Hartung *et al.*, 2006 ; Geuting *et al.*, 2009). De plus, le génome d'*Arabidopsis* contient deux orthologues de GEN1 (Osman *et al.*, 2011) mais leurs fonctions respectives restent à déterminer.

Projet de thèse

La réparation des cassures double brin de l'ADN par recombinaison est un processus fondamental au maintien de l'intégrité du génome. L'absence ou la mauvaise réparation d'une seule de ces lésions peut en effet avoir des conséquences dramatiques à l'échelle de la cellule, voire de l'organisme (mort cellulaire, tumorigenèse...). Dans une cellule sauvage, les CDB de l'ADN sont prises en charge par les voies de recombinaison homologue et non homologue. Chacune d'elle implique l'action coordonnée de nombreuses protéines, dont les fonctions sont extrêmement bien conservées.

La plante *Arabidopsis thaliana* constitue un modèle de choix pour l'étude des voies de recombinaison puisque des mutations dans les protéines clés n'affectent pas le développement végétatif de la plante, alors que ces mêmes mutations peuvent être extrêmement délétères chez les autres organismes. *Arabidopsis* permet donc l'étude des acteurs de la réparation des CDB à l'échelle de l'organisme, mais aussi les analyses d'épistasie puisqu'il est possible de générer des mutants multiples. Le génome d'*Arabidopsis* est de plus entièrement séquencé et de petite taille (environ 125 Mb répartis sur cinq chromosomes) (Arabidopsis Genome Initiative, 2000). Son cycle de vie relativement court et sa petite taille sont également des avantages pratiques intéressants. Enfin des banques de mutants d'insertion ont été générées et sont à la disposition de la communauté scientifique.

Les études précédemment menées au laboratoire ont consisté en l'analyse des protéines et des voies de la réparation des CDB induites par irradiation. Bien que les rayonnements ionisants produisent de multiples types de lésions au niveau de la molécule d'ADN, la réparation spécifique des CDB a pu être suivie grâce au marquage du variant d'histone γH2AX - un marqueur spécifique des CDB (Charbonnel *et al.*, 2010 ; Charbonnel *et al.*, 2011). L'analyse plus précise des protéines impliquées dans la réparation d'une CDB dans un contexte donné (séquence nucléotidique bordant la cassure, nature des extrémités d'ADN...) nécessite de simplifier le modèle d'étude en générant une lésion unique et localisée. Dans ce

contexte, l'objectif de ma thèse était d'analyser la contribution relative des différentes voies de recombinaison dans la réparation d'une CDB unique induite par une enzyme de restriction. Deux approches ont été menées en parallèle : la génération de mutants multiples de recombinaison portant des substrats chromosomiques rapporteurs de recombinaison et la mise au point d'une analyse en protoplastes grâce à l'expression transitoire de protéines fluorescentes, marqueurs des différentes voies de recombinaison.

L'analyse des lignées multiples mutantes a d'abord suggéré un rôle de la protéine XRCC2, un paralogue de RAD51, dans la voie d'hybridation simple brin (SSA) indépendante de RAD51. Or, seules des fonctions de cette protéine dans les voies de recombinaison homologue dépendantes de RAD51 avaient été décrites jusqu'alors chez les Eucaryotes (Shim *et al.*, 2004 ; Nagaraju *et al.*, 2009 ; Da Ines *et al.*, 2013a). Mes travaux se sont donc focalisés sur cette protéine, ainsi que sur deux autres paralogues de RAD51, RAD51B et RAD51D, et ont permis de démontrer leur implication dans la voie SSA (Article 1). Cette étude nous a mené à développer une analyse par Southern blot permettant de visualiser directement les produits de la recombinaison.

La seconde partie de ma thèse s'est focalisée sur le rôle du complexe XPF-ERCC1 dans les voies de recombinaison homologue, SSA et SDSA. Chez la levure comme chez les Mammifères, ce complexe est impliqué dans le clivage des extrémités d'ADN 3' non homologues des intermédiaires de recombinaison, mais cette fonction semble dépendante de la longueur des extrémités (Fishman-Lobell & Haber, 1992 ; Bardwell *et al.*, 1994 ; Ivanov & Haber, 1995 ; Pâques & Haber, 1997 ; Adair, 2000 ; Sargent *et al.*, 2000 ; Al-Minawi *et al.*, 2008). Nos analyses ont d'abord mis en évidence un rôle inhibiteur inattendu du complexe XPF-ERCC1 sur la recombinaison de type SDSA, et ont de plus confirmé la conservation des fonctions de ce complexe dans la recombinaison homologue chez la plante (Article 2).

Résultats

Chapitre I

Article 1: "Roles of XRCC2, RAD51B and RAD51D in RAD51-Independent SSA Recombination"

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Roles of XRCC2, RAD51B and RAD51D in RAD51-Independent SSA Recombination

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Abstract

The repair of DNA double-strand breaks by recombination is key to the maintenance of genome integrity in all living organisms. Recombination can however generate mutations and chromosomal rearrangements, making the regulation and the choice of specific pathways of great importance. In addition to end-joining through non-homologous recombination pathways, DNA breaks are repaired by two homology-dependent pathways that can be distinguished by their dependence or not on strand invasion catalysed by the RAD51 recombinase. Working with the plant *Arabidopsis thaliana*, we present here an unexpected role in recombination for the Arabidopsis RAD51 paralogues XRCC2, RAD51B and RAD51D in the RAD51-independent single-strand annealing pathway. The roles of these proteins are seen in spontaneous and in DSB-induced recombination at a tandem direct repeat recombination tester locus, both of which are unaffected by the absence of RAD51. Individual roles of these proteins are suggested by the strikingly different severities of the phenotypes of the individual mutants, with the *xrcc2* mutant being the most affected, and this is confirmed by epistasis analyses using multiple knockouts. Notwithstanding their clearly established importance for RAD51-dependent homologous recombination, XRCC2, RAD51B and RAD51D thus also participate in Single-Strand Annealing recombination.

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Introduction

DNA double-strand breaks (DSB) are produced by ionizing radiation, free radicals derived from metabolism, DNA cross-linking reagents and during DNA replication [1,2]. DSB can lead to mutations and rearrangements and/or loss of chromosomes, causing tumorigenesis or cell death. DSB must be repaired to maintain genome integrity, and this is carried out by end-joining through non-homologous recombination or by homologous recombination, which implicates DNA sequence homology of the recombining molecules (for reviews, see [3,4]). The pathways that utilize homology for repair can be distinguished by their dependence or not on strand-invasion catalysed by the RAD51 recombinase (or DMC1 in meiosis): gene conversion homologous recombination (HR) is RAD51-dependent while single-strand annealing (SSA) is RAD51-independent [3].

RAD51-dependent HR is an error-free DSB repair mechanism involving the use of a homologous template for restoration of the original sequence. It involves resection of the 5′-ended DNA strands at the DSB, generating 3′ single-stranded DNA overhangs that are bound by replication protein A (RPA). Assisted by mediator proteins, RAD51 displaces RPA and forms a helical nucleofilament on the exposed single-stranded DNA (ssDNA) flanking the DSB. This nucleofilament performs the homology search and catalyses invasion of the homologous template DNA, following which the invading 3′ ends are extended through DNA synthesis. The joint recombination intermediate is resolved to separate the recombining DNA molecules and thus restore chromosome integrity (for a review, see [3]).

In addition to RAD51 and the meiosis-specific DMC1, a number of RAD51 paralogue proteins have been described in a variety of organisms. These share 20% to 30% homology with RAD51 and presumably arose by gene duplication and evolved new functions [5]. They clearly play key roles in DNA repair through HR, but their exact functions are not fully understood (for reviews, see [6–8]).

Two *S. cerevisiae* RAD51 paralogues, RAD55 and RAD57, form a heterodimeric complex which associates with the RAD51 nucleoprotein filament, stabilising it against disruption by the SRS2 antirecombinase [9]. Recent work has characterized novel yeast RAD51 paralogues: Shu1, Shu2, Csm2 and Psy3, components of the "suppresses *sgs1* hydroxyurea sensitivity" (SHU or PCSS) complex which also promotes RAD51 filament assembly and its stability through counteracting the antirecombination activity of the SRS2 and SGS1 helicases [10–17]. Fission yeast has homologues of Shu1, Shu2 and Psy3 (Rlp1, Sws1 and Rdl1) and SWS1 and SWSAP1 are members of a human SHU complex [11,12,18].

Five RAD51 paralogues have been identified in animals and plants: RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 (for reviews, see [7,19,20]). Animal cells defective in any of the RAD51 paralogues are hypersensitive to DNA cross-linking agents, such as Cisplatin and Mitomycin C, and show spontaneous chromosomal aberrations [21–27]. Mouse *xrcc2*, *rad51b*, *rad51c* and *rad51d* mutants are embryonic lethal [28–31]. In contrast, all five RAD51 paralogues Arabidopsis mutants grow and develop normally and

Author Summary

The repair of DNA double-strand breaks by recombination is key to the maintenance of genome integrity in all living organisms. Recombination can however generate mutations and chromosomal rearrangements, making the regulation and the choice of specific pathways of great importance. Through modulation of the activity of the recombinase RAD51, the RAD51 paralogue proteins play key roles in the regulation of recombination. Considerable advances have been made in understanding of the RAD51 paralogue proteins and their roles in mediating RAD51-mediated homologous recombination, however very little is known of possible roles that they may have in other recombination pathways. Working with the plant, *Arabidopsis thaliana*, we show here major roles for three RAD51 paralogues in RAD51-independent single-strand annealing recombination. Notwithstanding their clearly established importance for RAD51-dependent homologous recombination, XRCC2, RAD51B and RAD51D thus also participate in Single-Strand Annealing recombination.

rad51c and *xrcc3* mutant plants are sterile due to recombination defects [32,33].

Two-hybrid and co-immunoprecipitation studies have shown that the five RAD51 paralogues form two major complexes: RAD51B-RAD51C-RAD51D-XRCC2 (BCDX2) and RAD51C-XRCC3 (CX3), as well as RAD51B-RAD51C (BC) and RAD51D-XRCC2 (DX2) sub-complexes [5,7,8,34–40]. RAD51 paralogue complexes act at both early and late stages of the recombinational repair process, although their exact roles remain to be identified [32,41–48]. The early role of RAD51 paralogues in HR is to promote formation and stabilization of RAD51 nucleoprotein filament (reviewed by [6–8,19]), very probably through counteracting disruption of the filament by helicases [9–14]. Recent work shows that the BCDX2 complex, and not the CX3 complex, is responsible for RAD51 recruitment at DNA damage sites in human cells [43]. After RAD51-mediated strand invasion, the RAD51 paralogues influence gene conversion tract length [42,47] and have been linked to Holliday junction (HJ) resolvase activity [45,46]. In addition, RAD51 paralogues can bind Y-shaped replication-like intermediates and synthetic HJ, in accordance with a role for RAD51 paralogues in repair during DNA replication and in resolution of HR intermediary structures [49,50].

The second main pathway using homology for repair, single-strand annealing (SSA), promotes recombination between tandemly repeated DNA sequences flanking a DSB. SSA does not involve DNA-strand invasion and has been shown to be independent of RAD51 [51–54]. After bidirectional 5′-3′ resection of the DSB ends, the exposed complementary sequences anneal. Subsequent removal of non-homologous 3′-ended ssDNA tails, filling-in of any single-strand gaps and ligation completes the process. The SSA recombination pathway thus leads to deletion of the interstitial DNA sequence lying between the repeats and one of the repeated homologous sequences (for reviews, see [3,55]).

Little is known about possible involvement of the RAD51 paralogues in RAD51-independent SSA. Yeast Rad55 and Rad57 are not required for SSA in a plasmid assay [51] or spontaneous direct repeat recombination [56,57] and a recent study has shown that absence of Rad55, Csm2 or Psy3 result in increased SSA recombination at a direct repeat chromosomal locus in yeast [15]. In Arabidopsis, RAD51, RAD51C and XRCC3 are not required for SSA, although a mild reduction in the efficiency of SSA was reported in the *rad51c* mutant [53].

In this study, we describe an unexpected role in the SSA pathway for Arabidopsis XRCC2, RAD51B and RAD51D, highlighting for the first time a function of these three RAD51 paralogues in RAD51-independent SSA recombination.

Results

XRCC2 is required for SSA recombination

Although XRCC2 is known to be involved in RAD51-dependent homologous recombination in both vertebrates and in plants [6,7,19,43,44], its potential role in RAD51-independent SSA has not been tested.

SSA recombination was monitored in *xrcc2* mutant *Arabidopsis thaliana* plants using the well-characterised DGU.US recombination reporter locus - consisting of an I-SceI restriction site flanked by 3′ and 5′ truncated copies of the β-glucuronidase gene (GUS) in direct orientation and with an overlap of 557 bp (Figure 1A; [58]). Cleavage of the I-SceI site induces recombination between the flanking GUS sequences and the resulting functional GUS gene is scored histochemically as blue somatic spots. I-SceI induced recombination at this tester locus has been shown not to depend upon RAD51 [53].

We introduced the GUS recombination reporter locus into *xrcc2* mutant and wild-type (WT) plants through crossing and transformed these DGU.US lines with an inducible I-SceI expression cassette (Materials and Methods). Three independent transformants (T2 lines) were selected for each genotype, each with a single insertion site of the I-SceI cassette. Seeds of these lines were plated onto medium containing hygromycin (in order to select plants carrying the I-SceI cassette), in the presence or absence of I-SceI expression inductor (β-estradiol), and numbers of blue GUS+ spots counted after 14 days of growth (Figure 1). Induction of I-SceI expression by β-estradiol treatment in WT plants resulted in a considerable increase of numbers of recombinant blue spots/sectors (Figure 1B and C). In contrast, expression of I-SceI had very little effect on numbers of blue spots in *xrcc2* mutant plants, with means of 5.9 spots per plant in the presence of β-estradiol, and 4.1 in its absence. Repetition of these analyses with two other independent I-SceI transformant lines yielded similar results (Figure 1D). XRCC2 thus clearly plays an important role in the SSA recombination pathway.

The histochemical GUS assay is an indirect measure of somatic recombination and we thus carried out Southern analyses to demonstrate directly that the decrease of number of GUS+ spots in *xrcc2* mutant plants is due to a failure of restoration of the GUS gene. Southern analysis was carried out on SacI-digested genomic DNA of WT and *xrcc2* mutant plants (induced or not by β-estradiol). In DGU.US lines, restoration of the GUS gene results in deletion of the repeated sequence, including the inserted I-SceI site. In DNA of WT plants, the reconstituted GUS gene is clearly visible as a band at the expected size (2.5 kb) after induction of I-SceI expression, but not in its absence (Figure 2, lanes 3 and 7). Treatment of the genomic DNA samples with I-SceI *in vitro* prior to electrophoresis confirms that the 2.5 kb fragment has lost its I-SceI site (Figure 2, lane 9), consistent with elimination of the I-SceI restriction site during the restoration of the marker gene. No restored GUS gene was detected in the *xrcc2* mutant line (Figure 2, lanes 8 and 10). This molecular analysis is thus fully consistent with the results of the β-glucuronidase assay and confirms the implication of the XRCC2 protein in the SSA recombination pathway.

We note also the presence of a 3.2 kb band in the SacI+I-SceI digested DNA from WT plants (Figure 2, lane 9). That this I-SceI resistant band is due to *in planta* rejoining of I-SceI breaks through

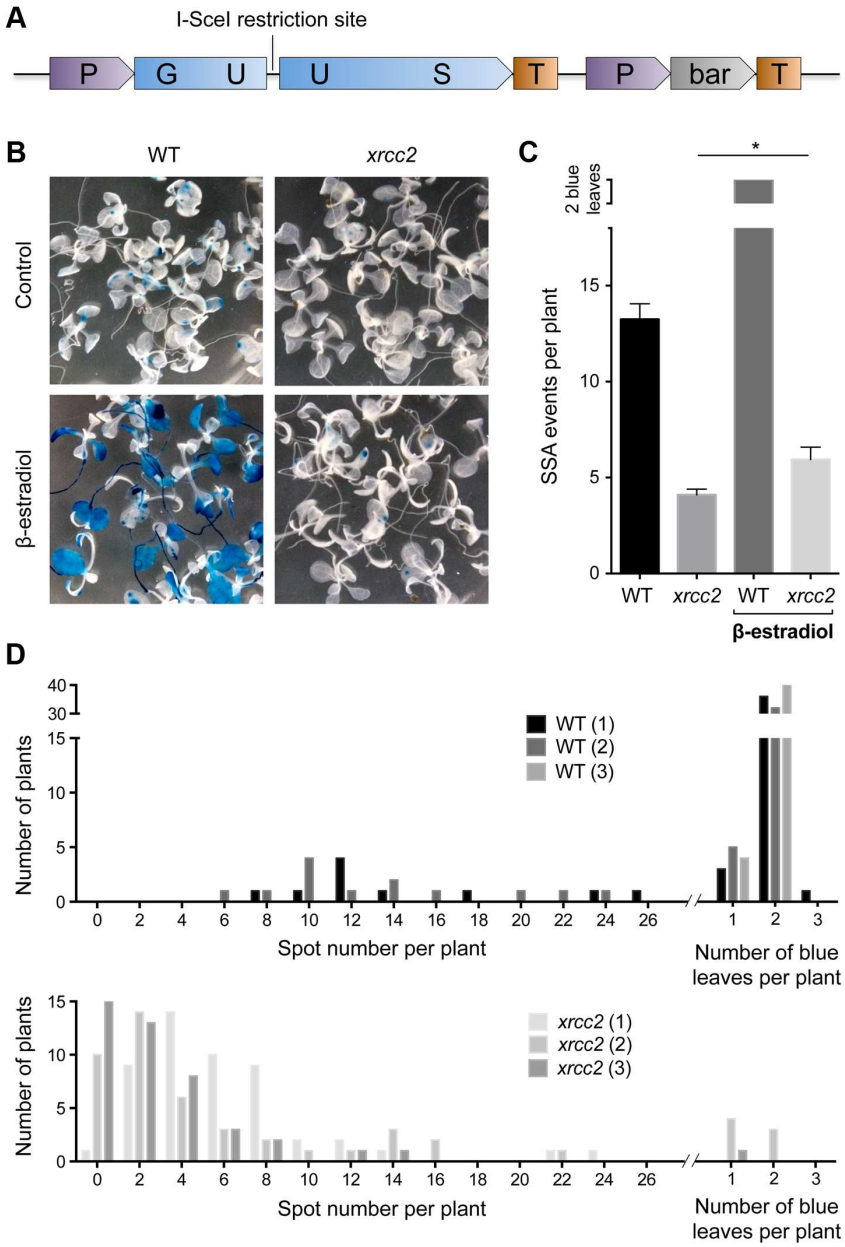

Figure 1. I-SceI induced DGU.US recombination depends upon XRCC2. (A) Schematic map of the recombination substrate DGU.US. (B) β-glucuronidase assay of 14 day-old seedlings grown with or without induction of I-SceI by β-estradiol clearly shows reduced numbers of blue recombinant GUS+ sectors in the *xrcc2* mutant. (C) Quantification of recombination events confirms the role of XRCC2. Bars are mean values ± standard errors. * Significant difference ($p = 0.036$, Mann-Whitney test). (D) Frequency distributions of recombinant spot numbers per plant of 3 independent WT and *xrcc2* T2 lines grown in the presence of β-estradiol.
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end-joining recombination was verified by PCR amplification and DNA sequencing. Approximately 10% of the sequences carried a mutation at the I-SceI restriction site. DNA sequencing showed that these result mostly from small deletions (Figure S1). As previously described [59–63], these events can be ascribed to end-joining exploiting the presence of microhomologies either side of the I-SceI cleavage site.

XRCC2 function in spontaneous DGU.US recombination does not depend upon RAD51 activity

Although minor, the DGU.US recombination analyses shown in Figure 1C also showed a difference in numbers of blue spots between WT and *xrcc2* plants in the absence of β-estradiol. To check whether this is due to differences in spontaneous recombination rates or to leakiness of the inducible I-SceI cassette (or

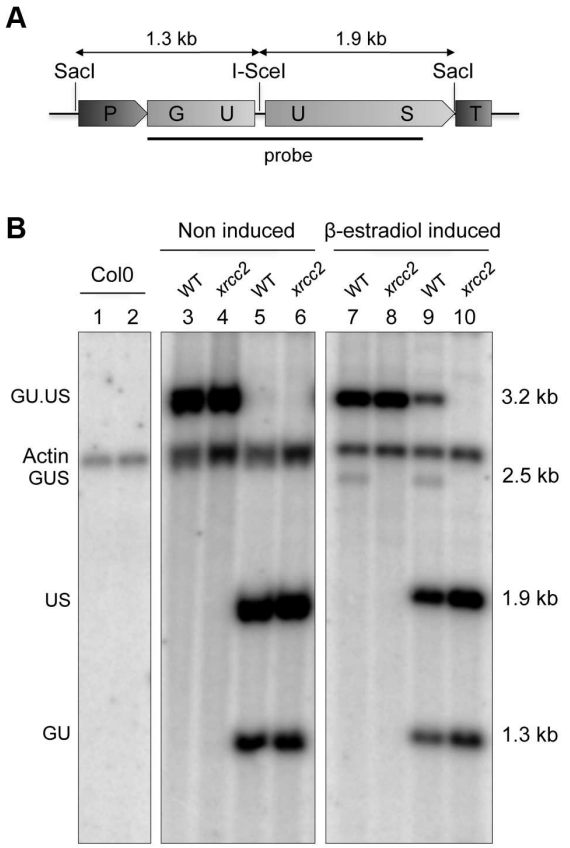

Figure 2. Molecular confirmation of recombination in WT, but not *xrcc2* mutant plants. Schematic representation of the GU.US recombination tester locus (A) and Southern analysis (B) of DNA from plants grown in the absence (lanes 1 to 6) or presence of β-estradiol (lanes 7 to 10), digested with SacI (lanes 1,3,4,7,8) or SacI plus I-SceI (lanes 2,5,6,9,10). The blot was hybridized with a GUS-specific probe as indicated in panel (A). The recombined GUS gene has lost its I-SceI site and is seen as a single 2.5 kb SacI fragment only in DNA from WT plants grown in presence of β-estradiol (lanes 7 and 9). Col0: WT plants of Columbia ecotype.
doi:10.1371/journal.pgen.1003971.g002

both), we monitored recombination in *xrcc2* mutant and WT plants with the same DGU.US locus (at the same location in genome), but which do not carry I-SceI (Figure 3). This analysis showed a reduction of number of recombinant spots in the absence of the I-SceI cassette, for both WT and *xrcc2* plants (from 13.24 to 5.46 and 4.10 to 0.36 spots per plant, respectively; Figures 1C and 3A), confirming the presence of some leakiness in expression of the I-SceI inducible promoter in the absence of β-estradiol. In the absence of the I-SceI cassette, mean numbers of blue spots per plant were however still significantly (15-fold) reduced in *xrcc2* mutants (0.36 ; s.e.m = 0.08) compared with WT controls (5.46 ; s.e.m = 0.34 ; Figure 3). An independent repetition of this experiment confirmed these results (Table 1). XRCC2 is thus clearly involved in spontaneous recombination of the DGU.US substrate.

As mentioned above, I-SceI induced recombination at the DGU.US locus has been shown to be RAD51-independent [53]. This has not however been confirmed for spontaneous recombination, for which different mechanisms can be envisaged - single-strand annealing, intermolecular synthesis-dependent strand annealing, break-induced replication [62,64,65]. We thus tested the RAD51-dependence of spontaneous recombination at DGU.US by expressing the dominant-negative RAD51-GFP fusion protein [66]. Plants were transformed with the RAD51-GFP fusion protein construct and three T2 lines each with a single insertion (RAD51-GFP plants) were selected and their *rad51* mutant phenotype tested by verification of sensitivity to the cross-linking agent, Mitomycin C (MMC) (Figure S2). Wild-type plants are not sensitive to the MMC dose used (2% sensitive plants), in contrast to the segregating RAD51-GFP population, in which 76.9% are sensitive. PCR genotyping confirmed that all of the MMC-sensitive and none of the MMC-resistant T2 plants carry RAD51-GFP. Presence of RAD51-GFP is thus perfectly correlated with MMC-sensitivity, confirming the dominant-negative inhibition of RAD51 by the fusion protein [66]. We then tested spontaneous DGU.US recombination in the RAD51-GFP plants (Figure 4). No significant difference was observed in numbers of GUS+ recombinant spots between control and RAD51-GFP plants (Mann-Whitney test) clearly confirming that spontaneous recombination of the DGU.US substrate does not depend upon RAD51 activity.

XRCC2, RAD51B and RAD51D have non-epistatic functions in the SSA pathway

XRCC2 is one of five RAD51 paralogue proteins, all of which play important roles in recombination [7]. Given the function of XRCC2 in the RAD51-independent SSA pathway presented above, we also tested for evidence of roles of the other RAD51 paralogues, RAD51B and RAD51D, in this pathway. We thus crossed the DGU.US recombination reporter locus into *rad51b* and *rad51d* mutant plants and monitored spontaneous SSA recombination at DGU.US in *rad51b* and *rad51d* mutants. Although less pronounced than the 15-fold reduction observed in *xrcc2* plants, numbers of spontaneous recombination events are also reduced in *rad51b* and *rad51d* mutants (respectively 4.6-fold and 3.4-fold; Figure 5; Table 2) clearly establishing roles for RAD51B and RAD51D in the SSA pathway.

Epistasis relationships in SSA recombination between the three RAD51 paralogue genes were tested in *xrcc2 rad51b* double and *xrcc2 rad51b rad51d* triple mutants. Spontaneous SSA recombination was significantly less efficient in *xrcc2 rad51b* double mutants that in the corresponding single mutants ($p<0.02$) (Figure 5). A slight further reduction in numbers of blue spots per plant was observed in the triple *xrcc2 rad51b rad51d* mutants with respect to the double *xrcc2 rad51b* mutant, but the difference is not significant.

To confirm at the molecular level the results of the GUS assays, we transformed *rad51b*, *rad51d*, *xrcc2 rad51b* and *xrcc2 rad51b rad51d* mutant plants with the inducible I-SceI expression cassette. Southern analysis of recombination was carried out on β-estradiol induced T2 plants. As expected, the 2.5 kb fragment of the recombination product is only detected in the WT, confirming the GUS assay data (Figure 6).

Arabidopsis XRCC2, RAD51B and RAD51D thus play roles in SSA recombination pathway and these roles are non-epistatic, at least for XRCC2 and RAD51B.

Discussion

The roles of RAD51 paralogues in RAD51-dependent recombination have been the subject of considerable interest in recent years [6–8,19]. Little is known however of possible roles in RAD51-independent SSA recombination. In Arabidopsis, no effect was found on SSA in *xrcc3* mutants and a barely statistically significant reduction observed in *rad51c* plants [53]. We show here the involvement of three RAD51 paralogues, XRCC2, RAD51B

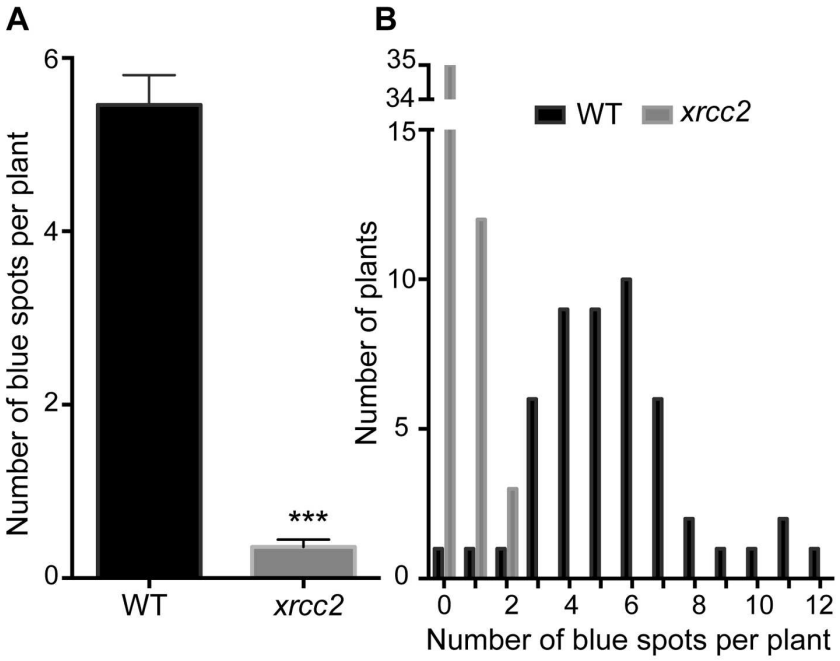

Figure 3. Spontaneous DGU.US recombination is reduced in the *xrcc2* mutant. A significant reduction in spontaneous recombination rate is observed in *xrcc2* mutant compared to WT plants. (A) Mean values ± standard errors of the means. *** $p<0.0001$ (Mann-Whitney test). (B) Frequency distributions.
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and RAD51D, in RAD51-independent single-strand annealing in *Arabidopsis thaliana*. XRCC2 plays a major role in this pathway with a striking reduction of I-SceI induced recombination and a 15-fold reduction in the number of spontaneous SSA events in its absence (Figures 1 and 3). Spontaneous SSA is also clearly reduced in *rad51b* and *rad51d* mutants (4.6-fold and 3.4-fold reduction respectively; Figure 5; Table 2), although less strongly than in *xrcc2* mutants. The differing severity of the phenotypes of the three mutants is suggestive of individual roles for these proteins, and this is supported by epistasis analyses of double and triple mutant plants (Figure 5, Table 2). An alternative to a direct role of these proteins is that the presence of non-functional RAD51 nucleofilaments in these mutants which might block SSA. The lack of effect on SSA of RAD51-GFP (which forms foci at DSBs and is dominant-negative for GC/SDSA recombination) however argues against this interpretation. Data suggesting differing roles for individual RAD51 paralogues, or sub-complexes, can be found in a number of reports. Individual paralogue mutants in DT-40 cells show non-epistatic phenotypes [67] and biochemical analyses show specific roles for the sub-complexes [68–70]. In Arabidopsis, absence of XRCC2 and RAD51B, but not RAD51D, increases rates of meiotic crossing-over [44] and RAD51D appears to be the only RAD51 paralogue to be essential for telomere integrity in human cells [71]. A recent report shows opposing effects on cell-cycle regulation of the inhibition of XRCC3 and RAD51C in HeLa cells, with inhibition of XRCC3 eliciting checkpoint defects and inhibition of RAD51C inducing G2/M cell cycle arrest [48].

Table 1. Spontaneous DGU.US recombination in *xrcc2* mutant and in wild-type plants.

Experiment		*n*	*N*	*m ± SEM*	*Ratio xrcc2/WT*
1	WT	50	273	5.46±0.34	
	xrcc2	50	18	0.36±0.08	**0.066**
2	WT	50	310	6.20±0.51	
	xrcc2	50	19	0.38±0.09	**0.061**

Recombination in the mutants and WT were compared using non-parametric statistical analysis (Mann–Whitney test). Differences between *xrcc2* and WT are highly significant ($p<0.0001$) in both cases. n, the number of plantlets screened; N, the total number of blue spots (recombination events) ; m ± SEM, the mean number of recombination events per plant ± standard error of the mean.
doi:10.1371/journal.pgen.1003971.t001

What can the roles of XRCC2, RAD51B and RAD51D be in the SSA pathway? The main steps of SSA are (1) bidirectional 5′ to 3′ resection of the DSB ends flanking a DSB, (2) annealing of exposed complementary sequences, (3) excision of non-homologous 3′-ended overhangs, (4) DNA synthesis and (5) ligation which restores two continuous strands [72,73]. A role in the annealing step is suggested by the capacity of the human BCDX2 complex to catalyse annealing between single-strand DNAs *in vitro* [50]. This study also showed a high affinity of the BCDX2 complex for branched DNA structures, such as Y-shaped DNA, that result from this annealing between tandem repeats during single-strand annealing. Taken together, these results strongly suggest a role of XRCC2, RAD51B and RAD51D in the annealing of the two exposed repeat sequences on either side of the DSB.

Biochemical studies have identified two main complexes of the five RAD51 paralogue proteins in animal and plant cells: RAD51B-RAD51C-RAD51D-XRCC2 and RAD51C-XRCC3 [5,7,8,34–40]. No self-assembly of individual RAD51 paralogues have been detected. Analysis of epistasis relationships of RAD51 paralogues in chicken DT-40 cells show that *rad51b* and *rad51d* are

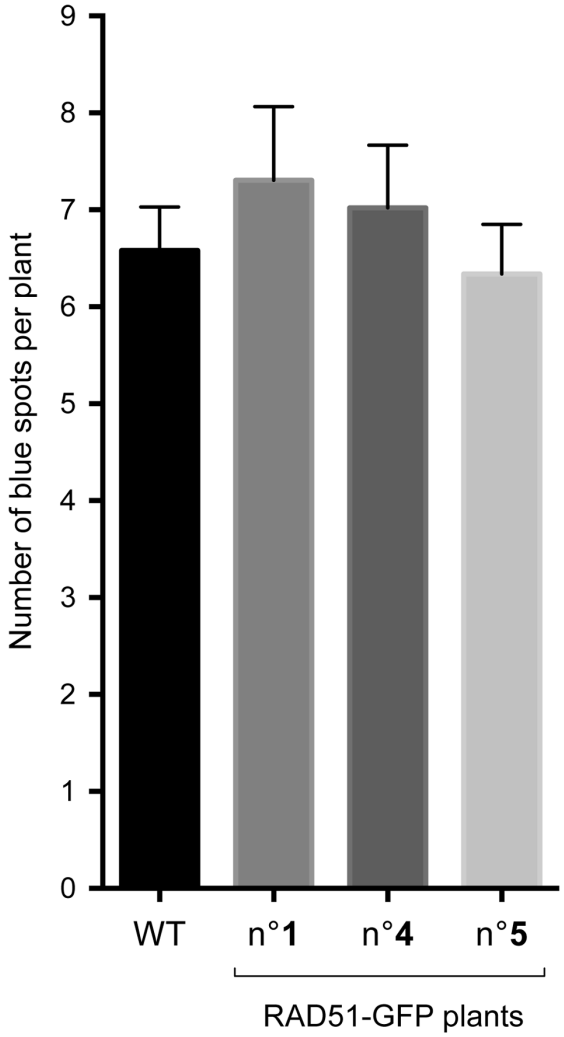

Figure 4. Spontaneous DGU.US recombination is RAD51-independent. No significant effect on spontaneous recombination rate was observed in three independent transformants carrying the dominant-negative RAD51-GFP construct. Bars are mean values ± standard errors.
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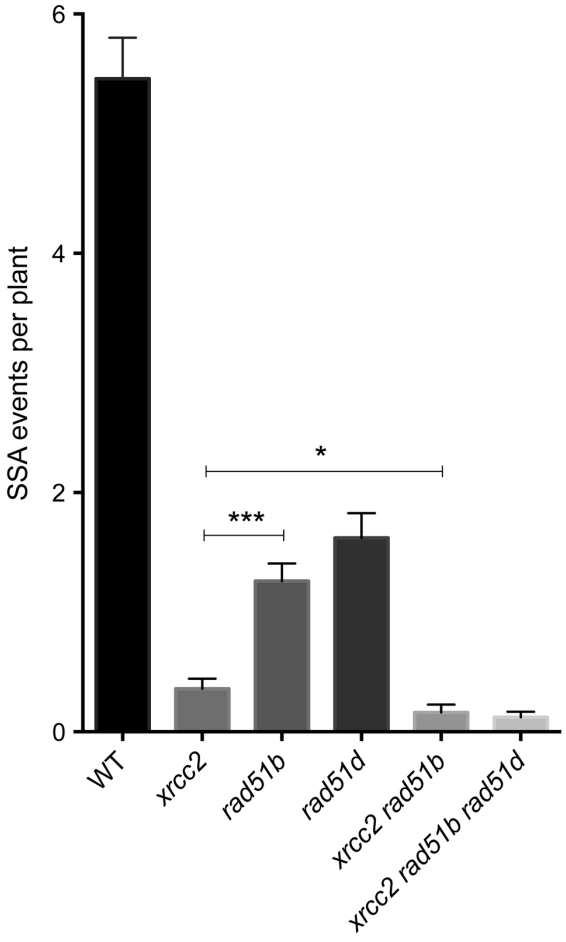

Figure 5. Individual and combined effects of *xrcc2, rad51b* and *rad51d* on spontaneous DGU.US recombination. Significant reductions in spontaneous recombination rate are observed in *xrcc2*, *rad51b* and *rad51d* mutants, and the severities of the reductions differ between these single mutants. A further significant reduction is seen in *xrcc2 rad51b* mutant plants. The triple *xrcc2 rad51b rad51d* mutant shows a further reduction, but this does not differ significantly from that observed in the *xrcc2 rad51b* plants. Bars are mean values ± standard errors. * $0.05<p<0.0001$; *** $p<0.0001$ (Mann-Whitney test).
doi:10.1371/journal.pgen.1003971.g005

epistatic while *xrcc3 rad51d* double mutant cells exhibit an additive sensitivity to ionizing radiation [67], consistent with differential actions of two major complexes in cellular response to DNA damage. That the three RAD51 paralogues involved in SSA are components of the BCDX2 complex suggests this complex is the active species in SSA. However, the differing severity of the phenotypes of the *xrcc2*, *rad51b* and *rad51d* (and *rad51c*; [53]) mutants argues against the implication of the BCDX2 complex as such. The proposed structure of the complex also argues against being the active form in SSA, with protein-protein interaction studies showing that the four proteins are linked in the order: RAD51B-RAD51C-RAD51D-XRCC2 [35]. Absence of RAD51D should thus exclude XRCC2 from the complex, yet SSA in the *xrcc2* mutants is significantly more affected than in *rad51d* (and similarly for *rad51b* versus *rad51c*). This argument also applies to the RAD51B-RAD51C and RAD51D-XRCC2 sub-complexes (for reviews, [7,8]). Our data thus favour individual roles of XRCC2, RAD51B and RAD51D in single-strand annealing recombination.

The yeast RAD51 paralogues Rad55 and Rad57 are not required in SSA recombination in a plasmid-based assay [51] and a chromosomal assay shows that absence of Csm2, Psy3 (also RAD51 paralogues) or Rad55 favours SSA with respect to gene conversion recombination [15]. The description here of roles for XRCC2, RAD51B and RAD51D in the RAD51-independent SSA pathway thus highlights a difference in the roles of Arabidopsis and yeast RAD51 paralogues in the SSA pathway. Such a difference is also seen in the roles of RAD51 paralogues in meiotic recombination with *psy3* mutants exhibiting a strong hypo-recombination in yeast [12], while absence of XRCC2 or RAD51B increases meiotic crossing-over in Arabidopsis [44].

In conclusion, we describe here an unexpected role in recombination for the Arabidopsis RAD51 paralogues XRCC2, RAD51B and RAD51D. The roles of these proteins are seen in

Table 2. Spontaneous DGU.US recombination in wild-type, *rad51b*, *rad51d*, double and triple mutants.

Experiment		*n*	*N*	*m ± SEM*	*Ratio mutant/WT*
1	WT	50	273	5.46±0.34	
	rad51b	50	63	1.26±0.15	**0.231**
	rad51d	50	81	1.62±0.21	**0.297**
	xrcc2, rad51b	50	8	0.16±0.07	**0.023**
	xrcc2, rad51b, rad51d	50	6	0.12±0.05	**0.022**
2	WT	50	310	6.20±0.51	
	rad51b	50	62	1.24±0.18	**0.200**
	rad51d	50	92	1.84±0.23	**0.297**
	xrcc2, rad51b	50	6	0.12±0.05	**0.019**
	xrcc2, rad51b, rad51d	50	1	0.02±0.02	**0.003**

Recombination in the mutants and WT was compared using non-parametric statistical analysis (Mann–Whitney test). Differences between each mutant and corresponding WT are highly significant ($p<0.0001$). n, the number of plantlets screened; N, the total number of blue spots (recombination events); m ± SEM, the mean number of recombination events per plant ± standard error of the mean.

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spontaneous and in DSB-induced recombination at a tandem direct repeat recombination tester locus, both of which are unaffected by the absence of RAD51. Notwithstanding their clearly established importance for RAD51-dependent homologous recombination, these proteins thus also participate in RAD51-independent Single-Strand Annealing recombination.

Materials and Methods

Plant material

The *Arabidopsis thaliana xrcc2*, *rad51b* [74] and *rad51d* [44] mutants used in this work have been previously described. A triple *xrcc2/xrcc2 rad51b/rad51b rad51d/rad51d* mutant was crossed with the recombination tester DGU.US-1 line [58] and single, double and triple mutants homozygous for the DGU.US substrate were identified in the F2. Wild-type control plants come from the same crosses.

The I-SceI coding sequence [75] was placed under control of β-estradiol in the plasmid pMDC7 [76] by Gateway cloning. The resulting vector was transferred into *Agrobacterium tumefaciens*, and used to transform the plant lines utilising the floral dip method [77].

Growth conditions

Surface-sterilized seeds were stratified at 4°C for 2 days and grown *in vitro* on germination medium (0.8% w/v agar, 1% w/v sucrose and half-strength Murashige & Skoog salts (M0255; Duchefa

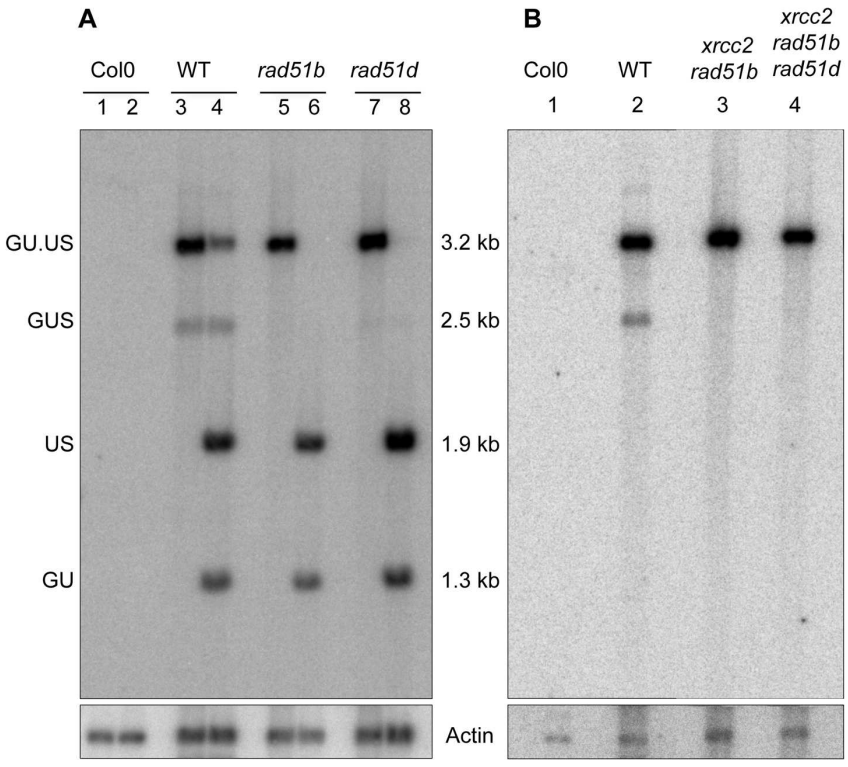

Figure 6. Molecular confirmation of recombination defects in *rad51b*, *rad51d*, *xrcc2 rad51b* and *xrcc2 rad51b rad51d* mutants. (A) Southern analysis of DNA from *rad51b* and *rad51d* mutant plants grown in the presence of β-estradiol, digested with SacI (lanes 1,3,5,7) or SacI plus I-SceI (lanes 2,4,6,8). (B) Southern analysis of DNA from *xrcc2 rad51b* and *xrcc2 rad51b rad51d* mutant plants grown in the presence of β-estradiol, digested with SacI. The blots were hybridized with a GUS-specific probe. The recombined GUS gene has lost its I-SceI site and is seen as a 2.5 kb band only in DNA from WT plants grown in presence of β-estradiol (A, lanes 3 and 4; B, lane 2). Col0: WT plants of Columbia ecotype.

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Biochemie, Netherlands)) in a growth cabinet with a 16-h light/8-h dark cycle, at 23°C with 45–60% relative humidity.

The growth medium was supplemented with 170 µM 17-β-estradiol (E2758; Sigma-Aldrich) for induction of I-SceI expression.

Histochemical staining of GUS expression

Fourteen-day old seedlings grown under standard conditions (supplemented or not with 17-β-estradiol) were harvested and incubated in staining buffer (0.2% Triton X-100, 50 mM sodium phosphate buffer (pH 7.2), 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; Biosynth), dissolved in N,N-dimethylformamide). Plants were infiltrated under vacuum for 15 min and incubated at 37°C overnight. The staining solution was then replaced with 70% ethanol to remove leaf pigments, and the blue spots were counted under a binocular microscope.

Mitomycin C treatment

Seeds were sown on plates containing fresh solid germination medium supplemented with 40 µM Mitomycin C (M0503; Sigma-Aldrich). The plates were then incubated for 20 days (23°C, 16-h light). A plant with 3 or more true leaves is considered resistant.

Plant DNA extraction and Southern analysis

DNA was prepared from seedlings as described previously [78] and 1.5 µg digested with 100 units of SacI or 25 units of I-SceI in a final volume of 200 µl for 15 h. Digested DNA samples were isopropanol precipitated, resuspended in TE, and electrophoresed in 0.8% agarose-TBE gel. Gel was blotted into a positively charged nylon membrane (Hybond-XL, Amersham Biosciences), which was hybridized in 0.5 M phosphate buffer, 7% w/v SDS, 1 mM EDTA (pH 8) and 1% BSA at 65°C. The DNA probes to the GUS gene (a PCR fragment amplified with 5′-TGGATCCC-CGGGATCATCTACTTCTG and 5′-AGCCATGCACACTG-ATACTCTTCACTCC) and the actin gene (a PCR fragment amplified with 5′-GGCTCCTCTTAACCCAAAGG and 5′-TT-ACCTGCTGGAATGTGCTG) were labelled with [α-^{32}P]dCTP using a random priming labelling kit (Megaprime DNA labelling system, Amersham) according to the manufacturer's instructions. Blots were washed with 0.5% SSC, 0.1% SDS solution at 65°C and imaged with a PhosphoImager (Bio-Rad Personal FX).

Supporting Information

Figure S1 I-SceI induced mutations in end-joining products of DGU.US repair. The unmodified sequence surrounding the I-SceI cut site of the DGU.US recombination tester locus is shown at the top of the alignment, with the I-SceI restriction site boxed and the cut-sites for each strand arrowed. Mutations are highlighted by gray boxes and the size of deletions (bp) is indicated at right. Flanking microhomologies presumably involved in the end-joining of I-SceI induced DSB are underlined.
(PDF)

Figure S2 Sensitivity to Mitomycin C in T2 Rad51-GFP plants. WT and three independent Rad51-GFP T2 transformants were tested for their sensitivity to the cross-linking agent MMC. The dominant-negative effect of the RAD51-GFP allele is clearly visible in the 3:1 segregating MMC hypersensitivity of the plantlets. (A) photos of the plantlets and (B) quantitation of sensitive versus resistant plants.
(PDF)

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Author Contributions

Conceived and designed the experiments: HS MEG CIW. Performed the experiments: HS ODI FD MEG. Analyzed the data: HS ODI FD MEG CIW. Contributed reagents/materials/analysis tools: MEG CIW. Wrote the paper: HS CIW.

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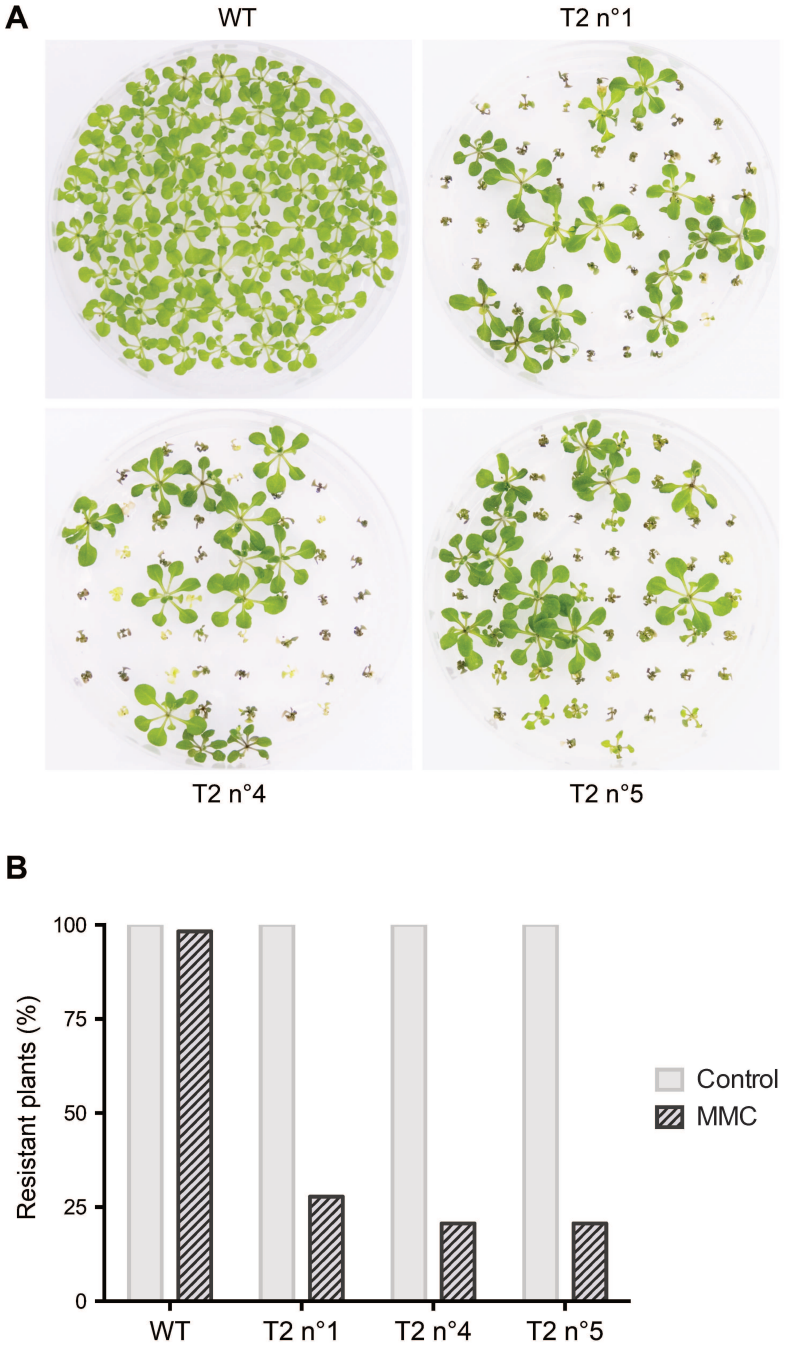
Figure S1


5' GUS fragment
I-SceI site
3' GUS fragment
GTGCCAGCGGCCGCCTAGGGATAACAGGGTAATAGTCTAGAGTCCTGTAG
cacggtcgccggcggatccctattgtcccattatcagatctcaggacatc
GTGCCAGCGGCCGCCTAGGGATA----------GTCTAGAGTCCTGTAG - 11
GTGCCAGCGGCCGCCTAGGGATAA---------TAGTCTAGAGCCCTGTAG - 8
GTGCCAGCGGCCGCCTAGGGATAA---------TAGTCTAGAGTCCTGTAG - 8
GTGCCAGCGGCCGCCTAGGGATAA---------TAGTCTAGAGTCCTGTAG - 8
GTGCCAGCGGCCGCCTAGGGATAA---------TAGTCTAGAGTCCTGTAG - 8
GTGCCAGCGGCCGCCTAGGGATAA---------TAGTCTAGAGTCCTGTAG - 8
GTGCCAGCGGCCGCCTAGGGATAA---------TAGTCTAGAGTCCTGTAG - 8
GTGCCAGCGGCCGCCTAGGGA---CAGGGTAATAGTCTAGAGTCCTGTAG - 3
GTGCCAGCGGCCGCCTAGG-ATAACAGGGTAATAGTCTAGAGTCCTGTAG - 1
GTGCCAGCGGCCGCCTAGGGATAACGGGGTAATAGTCTAGAGTCCTGTAG

Figure S2


A
WT
T2 n°1
T2 n°4
T2 n°5
B
Resistant plants (%)
100
75
50
25
0
WT
T2 n°1
T2 n°4
T2 n°5
Control
MMC

Chapitre I

Résultats complémentaires

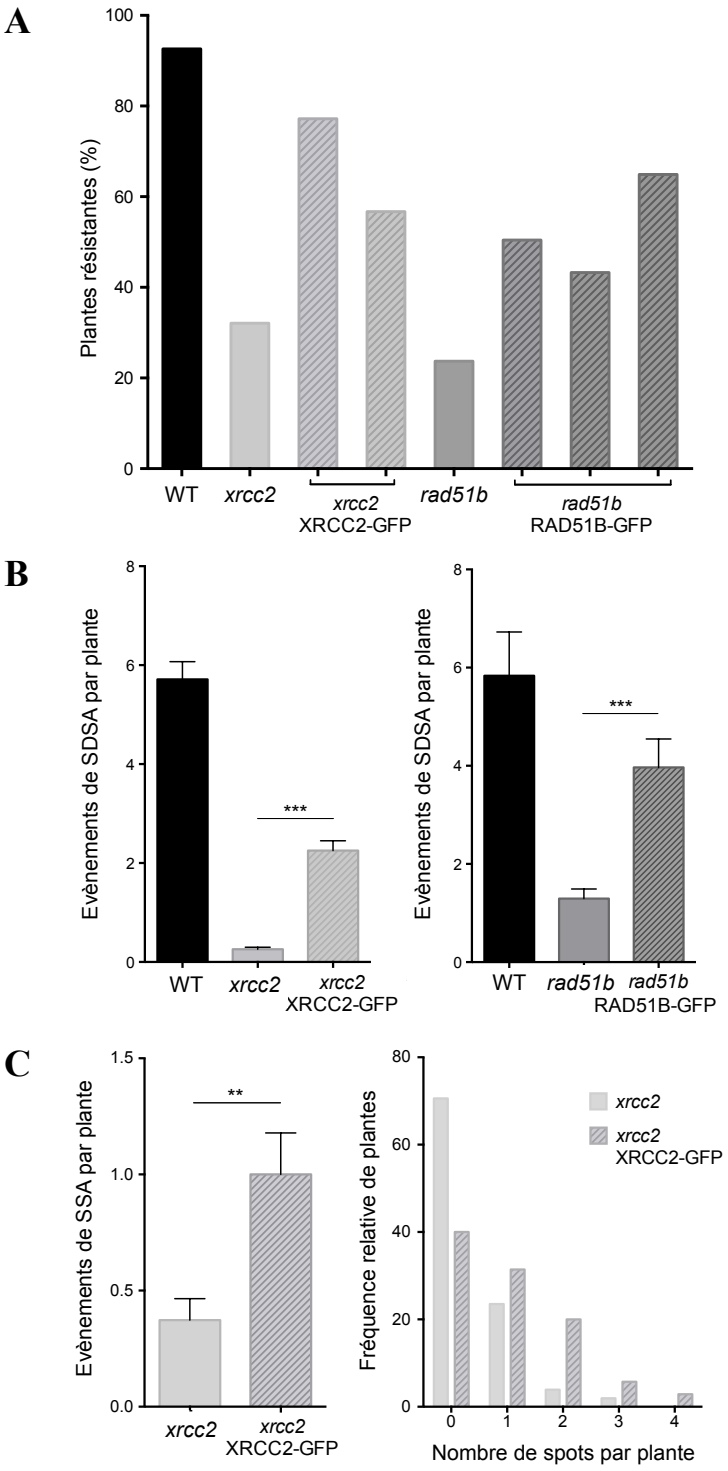


Figure 17 : Les protéines de fusion XRCC2-GFP et RAD51B-GFP complémentent la sensibilité à la Mitomycine C et les défauts de recombinaison des mutants *xrcc2* et *rad51b,* respectivement.

(A) Sensibilité à la MMC (40 µM) mesurée à 14 jours. Plusieurs lignées exprimant les protéines de fusion sont analysées. Une plantule avec au minimum 3 vraies feuilles est considérée résistante.

(B-C) Efficacité de la recombinaison de type SDSA au locus IU.GUS ou de la recombinaison de type SSA au locus DGU.US (cf Matériel et Méthodes, Article 1). Moyennes du nombre de spots recombinants par plante ± erreur standard. *** $p < 0{,}0001$; ** $p = 0{,}0015$ (test Mann-Whitney).

Dans cet article, nous avons mis en évidence un rôle de XRCC2, RAD51B et RAD51D - trois des cinq paralogues de RAD51 - dans la voie de recombinaison d'hybridation simple brin (SSA) chez la plante modèle *Arabidopsis thaliana.* Ce résultat était totalement inattendu puisque la recombinaison de type SSA est indépendante de RAD51 et seules des fonctions de ces paralogues dans la médiation de la stabilité et de l'activité de RAD51 ont été décrites jusqu'à présent (Brenneman *et al.*, 2002 ; Bleuyard & White, 2004 ; Liu *et al.*, 2004 ; Liu *et al.*, 2007 ; Nagaraju *et al.*, 2009 ; Rodrigue *et al.*, 2012 ; Chun *et al.*, 2013 ; Da Ines *et al.*, 2013a). En complément des résultats publiés dans l'Article 1, nous avons mené différentes expériences afin d'étayer et d'étendre les conclusions. Les résultats de ces expériences complémentaires sont présentés ci-dessous.

I. Validation du matériel d'étude

I.1. Vérification des mutants par complémentation

Afin de confirmer que les effets observés dans l'Article 1 sont strictement liés aux mutations des gènes des paralogues de RAD51, nous avons réalisé des expériences de complémentation des mutants avec des protéines de fusion générées au laboratoire. Les séquences génomiques de XRCC2 et RAD51B ainsi que leurs régions promotrices ont été amplifiées par PCR à partir de l'ADN d'une plante sauvage (d'écotype Columbia), puis fusionnées avec la séquence codante de la GFP. Les constructions XRCC2-GFP et RAD51B-GFP ont ensuite été introduites dans les plantes mutantes *xrcc2* ou *rad51b*, respectivement, par transformation selon la méthode d'immersion florale (Clough & Bent, 1998). L'analyse de la sensibilité de ces plantes à la Mitomycine C (MMC), un agent générant des ponts inter-brins dans l'ADN, a révélé que les protéines de fusion sont capables de complémenter la sensibilité des mutants correspondants (**Figure 17 A**). Dans les deux cas, la présence de la

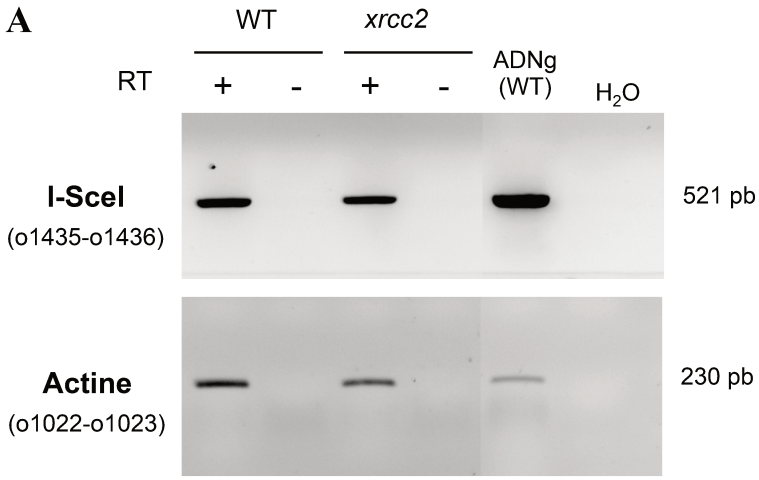
protéine de fusion (XRCC2-GFP ou RAD51B-GFP) sauvage restaure le phénotype sauvage des plantes mutantes (bien que partiellement dans certaines lignées).

Les lignées mutantes exprimant les protéines de fusion (*xrcc2* XRCC2-GFP et *rad51b* RAD51B-GFP) ont de plus été croisées avec des plantes mutantes possédant l'un des loci rapporteurs de recombinaison DGU.US ou IU.GUS (Orel *et al.*, 2003). La construction DGU.US est décrite dans la figure 1 de l'Article 1 et la construction IU.GUS (spécifique des évènements de CG/SDSA) sera présentée dans la figure 4 de l'Article 2. La quantification du nombre de spots recombinants par plante montre clairement que la présence des protéines XRCC2-GFP et RAD51B-GFP augmente l'efficacité de la recombinaison de type CG/SDSA (**Figure 17 B**) mais aussi du SSA (**Figure 17 C**) dans les plantes mutantes.

L'ensemble de ces résultats montre que la présence des protéines fonctionnelles complémente les phénotypes des mutants *xrcc2* et *rad51b* (sensibilité à la MMC et efficacité de la recombinaison). La restauration des phénotypes n'est cependant pas totale et varie dans les différentes lignées analysées. Ceci est probablement dû à la présence de l'étiquette GFP (27 kDa) et/ou aux différents niveaux d'expression des transgènes. Bien que la complémentation des phénotypes mutants avec les protéines sauvages soit dans certains cas partielle, ces expériences confirment que les taux faibles de recombinaison de type SSA dans les mutants *xrcc2* et *rad51b* sont liés à la mutation des gènes correspondants.

I.2. Vérification de l'induction des cassures double brin dans les plantes mutantes

L'efficacité de la recombinaison dans les plantes sauvages et mutantes pour XRCC2 a dans un premier temps été mesurée grâce à l'induction d'une CDB par l'enzyme de restriction I-SceI dans le locus DGU.US (Orel *et al.*, 2003). La cassette d'expression inductible d'I-SceI a été introduite dans chacune des lignées par transformation (*via Agrobacterium tumefaciens*) et les plantes présentant un seul site d'insertion ont été sélectionnées. Etant donné que le



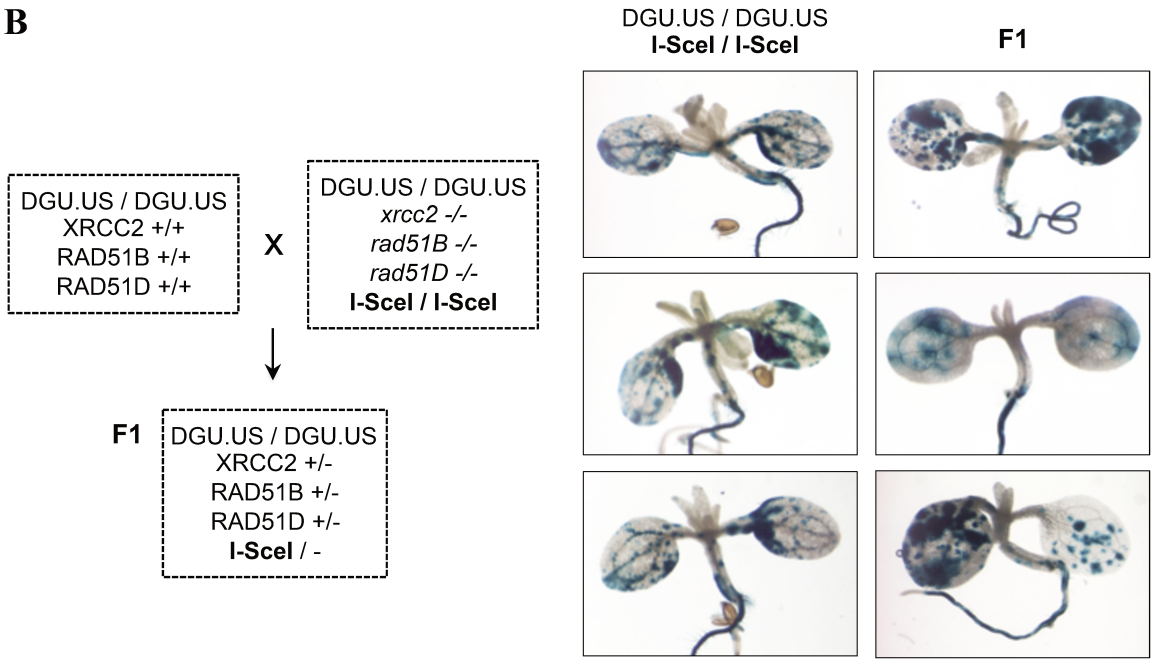


Figure 18 : La cassette d'expression inductible de l'enzyme I-SceI s'exprime dans les plantes mutantes *xrcc2* en présence d'inducteur et l'enzyme est fonctionnelle dans les mutants *xrcc2 rad51b rad51d.*

(A) Analyse de l'expression du gène I-SceI par RT-PCR à partir d'ARN extraits de plantes cultivées en présence d'œstradiol (l'inducteur de l'expression d'I-SceI). RT *(Reverse Transcription)*, ADNg (WT) : ADN génomique extrait de plantes sauvages possédant la cassette d'expression inductible d'I-SceI.

(B) Croisement de plantes sauvages avec des plantes mutantes *xrcc2 rad51b rad51d* exprimant I-SceI et mesure de l'efficacité de recombinaison des plantules F1 de 5 jours cultivées en présence d'œstradiol par révélation GUS (cf Matériel et Méthodes, Article 1).

transgène s'intègre de manière aléatoire dans le génome, le site d'insertion de la construction - et le contexte chromatinien - sont différents dans chacune des lignées sélectionnées. L'analyse de trois lignées indépendantes a donné des résultats similaires (**Article 1, figure 1 D**), ce qui suggère que le faible nombre de spots recombinants observés dans les mutants *xrcc2* n'est pas lié au site d'insertion de la cassette d'expression d'I-SceI. Afin d'étayer cette conclusion, deux analyses supplémentaires ont été menées pour vérifier l'expression du transgène et l'activité de clivage de l'enzyme I-SceI dans ces lignées (**Figure 18**). D'une part, l'expression de la construction dans une lignée de plantes sauvages et une lignée mutante *xrcc2* a été analysée par RT-PCR grâce à des amorces spécifiques de la séquence codante d'I-SceI. Cette analyse a confirmé que la quantité d'ARN messagers d'I-SceI est équivalente dans les deux lignées (**Figure 18 A**). D'autre part, pour confirmer que l'activité endonucléase d'I-SceI (au niveau de son site de clivage dans le locus DGU.US) est similaire dans les plantes sauvages et les plantes mutantes analysées, des mutants *xrcc2 rad51b rad51d* exprimant de manière inductible I-SceI ont été croisées avec des plantes sauvages (**Figure 18 B**). Ces deux lignées possèdent le substrat DGU.US à l'état homozygote. L'objectif est de vérifier l'activité de l'enzyme I-SceI du triple mutant en restaurant un contexte sauvage en F1 (un des allèles de chaque gène permet la production de chacun des paralogues de RAD51). Suite à l'induction de l'expression d'I-SceI, les plantules F1 présentent de nombreux spots et secteurs recombinants (**Figure 18 B**), contrairement aux plantules *xrcc2 rad51b rad51d* (où seuls quelques spots par plantes sont observés - donnée non montrée). La présence des paralogues de RAD51 fonctionnels est donc suffisante à la restauration du taux de recombinaison de plantules sauvages exprimant l'enzyme I-SceI (**Figure 18 B**). Ceci implique que le nombre de CDB générées dans le substrat DGU.US est équivalent dans les plantules sauvages et celles de la génération F1. Puisque le gène codant pour l'endonucléase responsable des cassures est

apporté par le génome des plantes *xrcc2 rad51b rad51d*, l'efficacité de clivage de l'enzyme I-SceI des plantules triples mutantes est donc équivalente à celle des plantules sauvages.

II. XRCC2 n'est pas impliqué dans la jonction des extrémités d'ADN

Pour confirmer à l'échelle moléculaire le rôle de XRCC2, RAD51B et RAD51D dans la recombinaison de type SSA, nous avons mis au point une analyse par Southern blot permettant de visualiser directement le produit de la recombinaison. Cette analyse a permis de démontrer que l'absence d'un ou plusieurs de ces trois paralogues de RAD51 abolit la capacité des cellules végétales à générer le produit de la recombinaison (**Article 1, figures 2 et 6**). L'ensemble des blots réalisés ont de plus mis en évidence l'existence d'une bande de 3,2 kb dans l'ADN des plantes sauvages préalablement digérées par I-SceI (**Article 1, figure 2, colonne 9 et figure 6 A, colonne 4**). Cette bande n'est visible que dans l'ADN des plantes cultivées en présence d'œstradiol (donc dans lesquelles l'enzyme I-SceI est exprimée) et est résistante à la digestion par I-SceI *in vitro*. L'amplification par PCR et le séquençage de ces fragments a révélé qu'une partie d'entre eux porte des mutations dans le site de restriction d'I-SceI (au niveau du substrat DGU.US), principalement des délétions de quelques nucléotides (**Article 1, figure supplémentaire 1**). Ces produits peuvent être attribués à des évènements de jonction des extrémités d'ADN (recombinaison non homologue), comme précédemment décrit dans la littérature (Salomon & Puchta, 1998 ; Heacock *et al.*, 2004 ; Puchta, 2005 ; Cermak *et al.*, 2011 ; Miller *et al.*, 2011). Ces fragments ne sont en revanche jamais détectés dans l'ADN de plantes mutantes *xrcc2* (**Article 1, figure 2**). Ceci suggère que la protéine XRCC2 pourrait jouer un rôle dans la jonction des deux extrémités d'ADN issues de la digestion par l'enzyme I-SceI (extrémités cohésives 3' sortantes).

Afin de tester cette hypothèse, nous avons utilisé un test *in vivo* de recircularisation d'un plasmide mesurant la capacité des cellules végétales à réparer une CDB générée par

A

		Nombre de noyaux	Noyaux **YPF+**	
			Pourcentage	Nombre relatif
-	WT	2301	0,1 %	0,2
	WT	2094	51,1 %	100
	xrcc2	1964	61,8 %	100
	WT	1197	2,3 %	**4,4**
	xrcc2	1932	3,2 %	**5,1**
	WT	1672	0,4 %	0,7

B

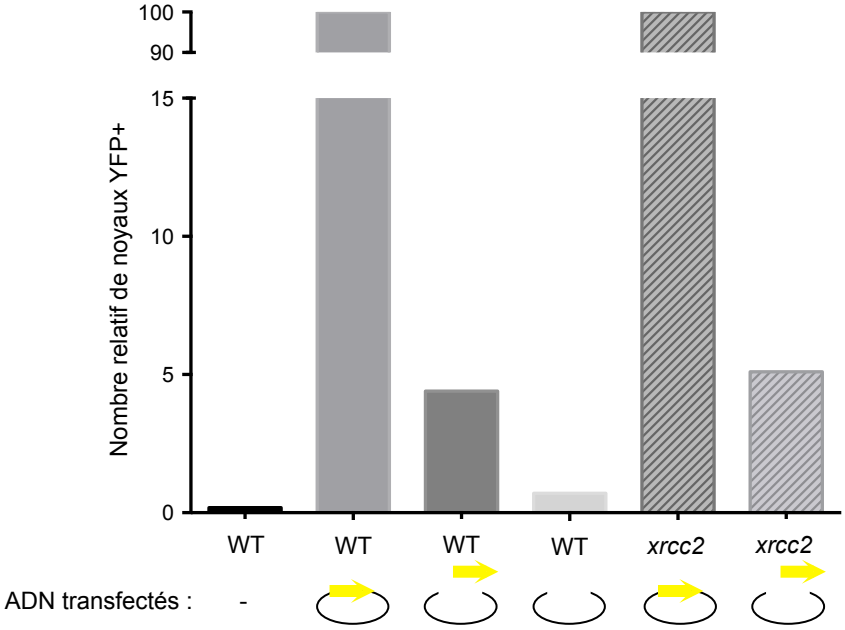


Figure 19 : XRCC2 n'est pas impliqué dans la jonction des extrémités d'ADN.

Un plasmide contenant le gène de fusion H2B-YFP sous le contrôle du promoteur 35S (préalablement digéré ou non par l'enzyme I-SceI) est transfecté dans des protoplastes d'*Arabidopsis* sauvages ou mutants pour XRCC2. Le plasmide linéaire sans le fragment H2B-YFP-T_{NOS} (représenté par un rectangle jaune) est également transfecté dans des protoplastes sauvages comme contrôle négatif. 24h après transfection, les noyaux sont extraits des protoplastes et la fluorescence jaune analysée par cytométrie en flux.

l'enzyme de restriction I-SceI. Des tests similaires ont précédemment été utilisés dans des protoplastes ou des feuilles de tabac et d'*Arabidopsis thaliana* (Gorbunova & Levy, 1997 ; Gorbunova *et al.*, 2000 ; Gallego *et al.*, 2003). Le plasmide utilisé contient la séquence codante de l'histone H2B fusionnée à celle du gène rapporteur YFP, sous le contrôle du promoteur 35S. Un site de restriction de l'enzyme I-SceI est situé entre le promoteur 35S et la séquence codante H2B et un autre se situe en aval du terminateur du gène de fusion (terminateur de la nopaline synthétase, T_{NOS}). La digestion du plasmide par I-SceI excise donc le fragment H2B-YFP-T_{NOS}. Des protoplastes issus de feuilles de plantules d'*Arabidopsis* sauvages et mutantes *xrcc2* sont transfectés avec le plasmide circulaire (contrôle de transfection) ou le plasmide digéré *in vitro* par I-SceI (plasmide linéarisé + fragment H2B-YFP-T_{NOS}). La réintégration du fragment H2B-YFP-T_{NOS} dans le plasmide linéarisé au sein des protoplastes permet l'expression du gène de fusion. La présence de la protéine nucléaire H2B-YFP dans les protoplastes est détectée par cytométrie en flux après extraction des noyaux (24h après transfection). Davantage de détails concernant le plasmide utilisé, l'obtention et la transfection des protoplastes ainsi que l'analyse par cytométrie en flux sont présentés dans le chapitre III.

Les résultats présentés dans la **figure 19** indiquent que l'efficacité de transfection des protoplastes sauvages et *xrcc2* est relativement similaire (51,1 % et 61,8 % de protoplastes fluorescents YFP+, respectivement). La capacité des cellules végétales à réparer les CDB générées par I-SceI est mesurée par le nombre de protoplastes YFP+ après transfection du plasmide digéré, normalisé au nombre obtenu avec le plasmide circulaire. Nos résultats montrent que cette capacité est équivalente dans les deux fonds génétiques (**Figure 19**), indiquant que la protéine XRCC2 n'est pas requise à la jonction des extrémités d'ADN dans ce système plasmidique. Ces analyses suggèrent donc que XRCC2 n'est pas impliqué dans les

voies de jonctions des extrémités d'ADN chez Arabidopsis, comme c'est le cas chez les Mammifères (Johnson *et al.*, 1999).

III. XRCC2 joue un rôle indépendant de RAD51 dans la voie SSA

Les analyses menées dans cet article ont démontré que les protéines XRCC2, RAD51B et RAD51D sont impliquées dans la voie de recombinaison d'hybridation simple brin (SSA). La capacité du complexe BCDX2 humain à catalyser l'hybridation de fragments d'ADNsb *in vitro* suggère un rôle direct de ces protéines dans l'étape d'hybridation des deux séquences répétées (Yokoyama *et al.*, 2004). Cette étude a de plus montré que le complexe BCDX2 présente une haute affinité pour les structures d'ADN branchées, telles que les structures d'ADN en Y qui résultent de l'hybridation des séquences répétées au cours du SSA. Bien que ces résultats soutiennent l'hypothèse d'un rôle direct de XRCC2, RAD51B et RAD51D dans la voie SSA, une action indirecte de ces protéines ne peut pas être exclus. Etant donné que les paralogues de RAD51 sont impliqués dans la formation et la stabilisation des filaments nucléoprotéiques RAD51-ADNsb (pour revue, voir Suwaki *et al.*, 2011), il est possible que l'absence d'un paralogue conduise à la formation d'un filament non-fonctionnel. La présence de ce filament pourrait bloquer l'hybridation des séquences répétées situées de part et d'autre de la cassure par encombrement stérique. Ainsi, la voie SSA serait inhibée de manière indirecte en absence d'un ou de plusieurs paralogues de RAD51.

Afin de tester ces hypothèses, nous avons généré des plantes mutantes pour XRCC2 et RAD51 possédant le locus rapporteur de recombinaison DGU.US (Orel *et al.*, 2003). Ces plantes sont issues du croisement d'une lignée homozygote pour le substrat DGU.US et pour la mutation *xrcc2* avec une lignée homozygote pour le même substrat, pour un allèle KO *rad51* et pour la construction RAD51-GFP. La protéine de fusion RAD51-GFP restaure la fertilité du mutant *rad51* indispensable à la réalisation des croisements (Da Ines *et al.*, 2013b).

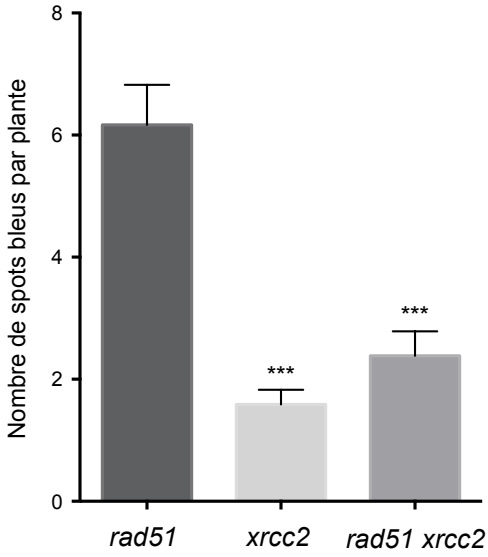


Figure 20 : La réduction de l'efficacité du SSA observée en absence de XRCC2 est indépendante de RAD51

L'efficacité de la recombinaison de type SSA au locus DGU.US est mesurée comme décrit dans l'Article 1 (Matériel et Méthodes). Moyennes du nombre de spots recombinants par plante ± erreur standard. Le nombre moyen de spots recombinants dans les lignées *xrcc2* et *rad51 xrcc2* n'est pas significativement différent. *** $p \leq 0{,}0001$ (test Mann-Whitney).

L'efficacité de la recombinaison de type SSA est mesurée dans des plantes *xrcc2*, *rad51* et *xrcc2 rad51* qui ne possèdent pas la construction RAD51-GFP (**Figure 20**). Comme attendu, le nombre moyen de spots recombinants par plante est significativement réduit dans les mutants *xrcc2* par rapport aux mutants *rad51* - l'efficacité du SSA étant équivalente dans des plantes sauvages et des plantes dont RAD51 est non-fonctionnel (**Article 1, Figure 4**). L'efficacité de la réparation dans le double mutant *xrcc2 rad51* n'est en revanche pas significativement différente de celle du simple mutant *xrcc2*, indiquant que l'inhibition du SSA en absence de la protéine XRCC2 est indépendante de la présence de la protéine RAD51. Ces résultats démontrent donc que XRCC2 joue un rôle indépendant de RAD51 - très probablement direct - dans la voie SSA. Cette étude constitue, à notre connaissance, la première démonstration d'une fonction indépendante de RAD51 d'un paralogue de RAD51. Des études biochimiques seront nécessaires à l'identification de son rôle précis.

Chapitre II

Article 2: "A role for XPF-ERCC1 in inhibiting homologous recombination"

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Maria E. Gallego & Charles I. White

(article soumis)

ABSTRACT

In addition to its well-characterized role in nucleotide excision repair, the XPF-ERCC1 (Rad1-Rad10 in yeast) structure-specific endonuclease plays an important role in recombination. Cleaving DNA at double-strand to single-strand junctions, XPF-ERCC1 acts to remove non-homologous single-stranded 3' tails from homologous recombination intermediates. The need for this activity however depends on both the length and the number of the non-homologous tails. Using recombination substrates which generate only short non-homologous tails, and thus should not require the action of XPF-ERCC1, we have taken advantage of this to further study the roles of this endonuclease in recombination in the plant, *Arabidopsis thaliana*.

Using a specific reporter for RAD51-dependent recombination we show that both spontaneous and I-SceI induced recombination are significantly enhanced (more than 2-fold) in *xpf* mutants relative to the WT controls. Expression of a wild-type XPF-GFP fusion protein in *xpf* mutant plants supresses the hyper-recombination phenotype, confirming that it specifically results from the absence of XPF. In contrast, expressing the nuclease-dead XPF_{D757A}-GFP protein in *xpf* mutant plants does not affect the hyper-recombination effect of the absence of XPF.

The endonuclease activity of XPF thus acts to inhibit RAD51-dependent homologous recombination and we propose that this results from effects on homology search *in cis*, versus *in trans*, in a repair pathway competition model.

AUTHOR SUMMARY

XPF-ERCC1 is a key actor in the repair of DNA through nucleotide excision and the importance of its role is seen in patients with the cancer-prone Xeroderma Pigmentosum syndrome. In addition to this role in nucleotide excision repair, the XPF-ERCC1 structure-specific endonuclease plays a well-characterized role in recombination and the repair of DNA double-strand breaks. In order to search for further roles of this endonuclease, we present here a study involving specific tester loci which do not require the action of XPF-ERCC1 for recombination. Absence of XPF endonuclease activity results in an enhancement of RAD51-dependent homologous recombination in *Arabidopsis thaliana*. XPF thus acts to inhibit homologous recombination and we propose that it does so by favouring the use of local micro-homologies and thus restricting the search for more distant homologous donor sequences.

INTRODUCTION

The efficient repair of damaged DNA is essential for the maintenance of genomic integrity of all living organisms. Living cells have evolved multiple ways of repairing the many types of DNA damage thanks to the coordinated action of numerous proteins (for reviews, see [1,2]). This work concerns one of these proteins - the XPF *(xeroderma pigmentosum complementation group F)* protein. Disruption of XPF gene has dramatic consequences in mammals: XPF-deficient mice show growth retardation and die approximately 3 weeks after birth [3] and mutations of human XPF result in the UV sensitive cancer-prone syndrome, *Xeroderma Pigmentosum* [4]. Consistent with its essential role in DNA damage repair, XPF is very well conserved in eukaryotes, with well-studied orthologues in many organisms including Rad1 in *Saccharomyces cerevisiae* [5], Rad16 in *Schizosaccharomyces pombe* [6], Xpf-1 in *Caenorhabditis elegans* [7], Mei-9 in *Drosophila melanogaster* [8] and UVH1/RAD1/XPF in *Arabidopsis thaliana* [9-12].

The XPF protein forms a heterodimeric complex with ERCC1 *(excision repair cross-complementation 1)* protein *in vitro* and *in vivo* [4] and formation of this complex stabilises the two proteins [13-15]. The XPF-ERCC1 complex and its yeast homologue Rad1-Rad10 are structure- specific endonucleases which cleave DNA at double strand (ds) to single strand (ss) junctions, nicking the dsDNA on the 5'-3' strand, two nucleotides from the junction [4,16-18]. *In vitro* endonuclease assays revealed that XPF-ERCC1 cleaves a variety of substrates, including stem- loops and structures with 3' overhangs [16]. XPF provides the endonuclease activity of the complex, while ERCC1 is catalytically inactive but instead regulates DNA- and protein-protein interactions [19-21].

XPF-ERCC1 was first identified as a factor required for nucleotide excision repair (NER) of helix-distorting DNA lesions, especially ultra-violet (UV)-induced damage [4,11,15]. The complex is required for 5' cleavage of the DNA lesion in DNA near sites of

damage (for reviews, see [22,23]). XPF-ERCC1 is also involved in the initial step of interstrand crosslinking (ICL) repair pathway, making the incisions on both sides of an ICL placed near a dsDNA-ssDNA junction [24]. Moreover, the XPF-ERCC1 complex has been found to function in homology-directed double- strand break (DSB) repair, through single-strand annealing (SSA) and gene conversion (GC) [17,25-31].

SSA is a major recombination pathway for repairing DSB that arise between repeated sequences [32,33]. After bidirectional 5'-3' resection of the DSB ends, RAD52 (and Rad59 in budding yeast) promotes the annealing of the exposed complementary sequences. The 3' ssDNA ends are non-homologous with the new flanking regions, and must be cleaved in order to complete repair by filling-in of any single-strand gaps and strand ligation (for reviews, see [32,34]). The removal of the non-homologous 3' tails is ensured by XPF-ERCC1 (Rad1-Rad10) endonuclease in association with MSH2-MSH3 mismatch recognition complex [17,26,27,30,31,35,36]. Two additional actors have been identified in yeast: Slx4 *(Synthetic lethal of unknown function protein 4)* and Saw1 *(Single-strand annealing weakened 1)* [37,38]. Saw1 facilitates targeting of Rad1 at 3' tailed substrates and enhances the cleavage activity of Rad1-Rad10 [39], and Slx4 is a scaffold for the binding of a number of DNA repair proteins, including XPF-ERCC1 [40-42].

Homologous recombination by gene conversion (GC) can also involve the removal of 3' non-homologous tails. The most common models of GC are the synthesis dependent strand annealing (SDSA) [43], the double strand break repair (DSBR) [44] and the break induced replication (BIR) models [45,46]. Most mitotic GC events are thought to occur by the SDSA mechanism [32,47,48]. In this model, the 5'-3' resection of the DSB ends generates 3' ssDNA overhangs that are bound by RAD51 and the resulting nucleofilament is the active molecular species in searching for and invading a homologous donor DNA sequence. Before an invading 3' DNA end can prime new DNA synthesis to copy the donor template, any non-

homologous 3' tail must however be removed. Subsequent unwinding of the extended invading strand from the donor DNA duplex allows it to then anneal back to the non-invading DSB end. Removal of the second non-homologous 3' tail is then potentially required to permit gap filling synthesis and ligation, which complete the process (for reviews, see [32,34,35]). If only one side of the DSB ends with non-homologous sequence, the 3' end that shares homology with the donor sequence can perform the strand invasion and there is no barrier to the initiation of DNA synthesis. Numerous studies have demonstrated the involvement of XPF-ERCC1 (Rad1-Rad10) complex in the removal of non- homologous 3' tails during GC in yeasts, animals and plants [17,25,26,28-31,35]. The requirement for XPF-ERCC1 (Rad1-Rad10) during both SSA and GC processes depends not only on the presence of non-homologous 3' tails, but also on their length. Neither Rad1-Rad10 in yeast, nor XPF-ERCC1 in mammals, is required for GC or SSA if non-homologous 3' tails are less than approximately 30 nucleotides long [25,27,30,31,49]. In plants, the roles of Arabidopsis XPF and ERCC1 in SSA and GC involving the removal of long non-homologous tails have been confirmed using plasmid and chromosomal substrates [28,29] and in this work we confirm that Arabidopsis XPF is not required for the removal of short non-homologous 3' tails in both SSA and SDSA recombination, as is the case for recombination in yeasts and animals.

The use of recombination substrates with only short non-homologous overhangs has permitted us to bring to light an unexpected hyper-recombination phenotype in *xpf* mutant plants. We show that this is due to a role of the nuclease activity of XPF in inhibiting SDSA recombination and propose that it results from effects on homology search in a repair pathway competition model.

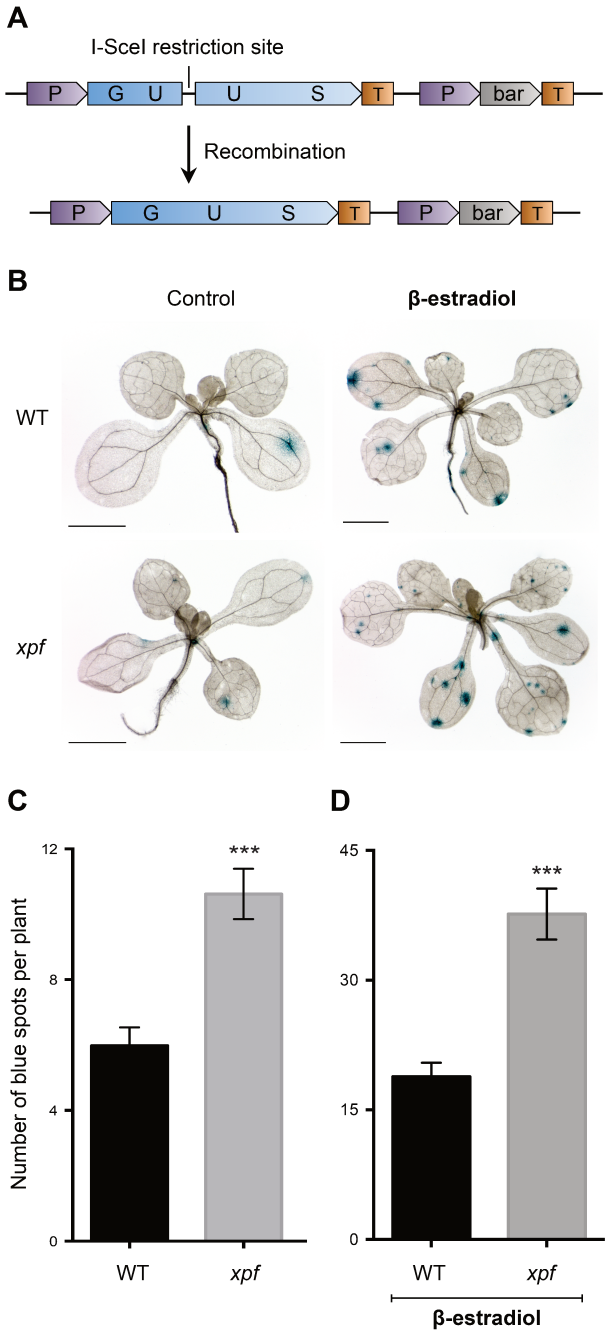
A
I-SceI restriction site
P
G U
U
S
T
P
bar
T
Recombination
P
G
U
S
T
P
bar
T
B
Control
β-estradiol
WT
xpf
C
Number of blue spots per plant
12
8
4
0
***
WT
xpf
D
45
30
15
0
***
WT
xpf
β-estradiol

Figure 1

RESULTS

XPF is not required for the removal of short non-homologous ends during SSA

Neither yeast Rad1-Rad10, nor mammalian XPF-ERCC1 is required for removal of short non-homologous tails during homologous recombination [25,27,30,31,49]. In the course of our studies of the roles of structure-specific endonucleases in recombination, we tested in Arabidopsis this XPF-independence of recombination involving short non-homologous overhangs using the well-characterised chromosomal DGU.US recombination reporter locus. This consists of an I-SceI restriction site flanked by 3' and 5' truncated copies of the β-glucuronidase gene (GUS) in direct orientation and with a sequence homology overlap of 557 bp (**Figure 1**) [50]. DNA cleavage by I- SceI expression *in planta* induces recombination between the flanking GUS sequences and the resulting functional GUS gene is scored histochemically as blue spots. In the case of I-SceI induced DSB, repair by SSA involves the removal of two 3' single stranded DNA ends of 21 nucleotides. The situation is more complex in the case of spontaneous DSB: if the break occurs between the two repeated sequences, SSA repair would involve the removal of two short tails, however repair of a break in one of the repeated sequences would involve the removal of only one 3' tail.

The GUS reporter locus was introduced into *xpf* mutant and wild-type (WT) plants through crossing, followed by further crosses to introduce the inducible I-SceI expression cassette (Materials and Methods). Recombination efficiency was monitored in 14 day-old DGU.US WT and *xpf* mutant plants carrying, or not, the I-SceI expression cassette (**Figure 1B, C, D**). The data show clearly that the absence of XPF causes no reduction in efficiencies of spontaneous nor of I-SceI induced DGU.US recombination - to the contrary, *xpf* mutant plants are significantly hyper- recombinant at this locus. An independent repetition of this experiment gave similar results (**Table 1**). These data thus confirm that XPF is not required

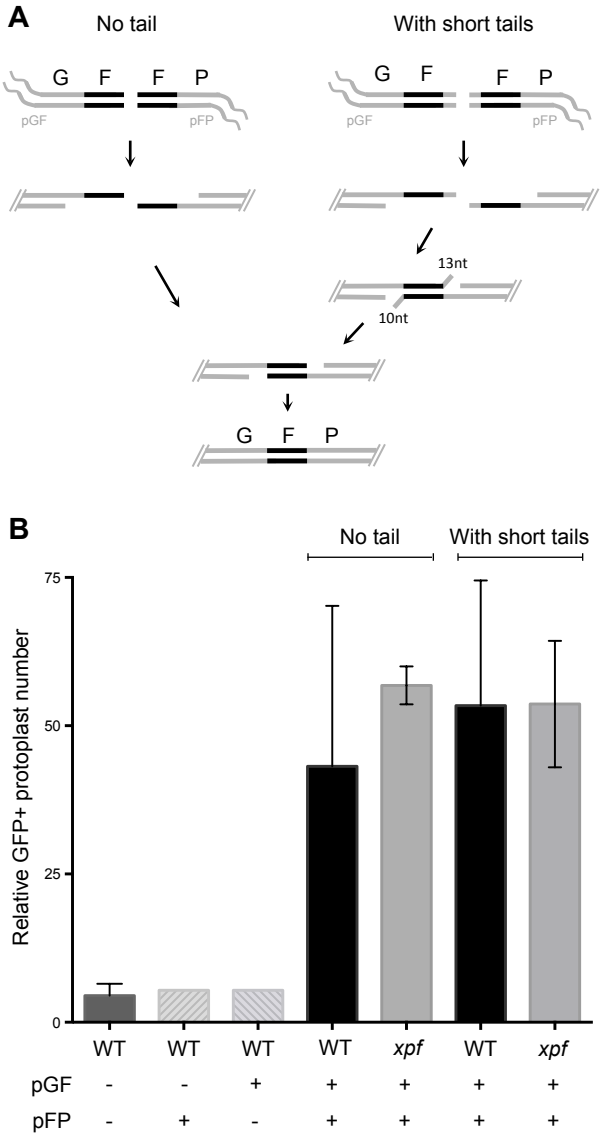
A
No tail
With short tails
G F F P
pGF
pFP
13nt
10nt
G F P
B
No tail
With short tails
Relative GFP+ protoplast number
75
50
25
0
WT WT WT WT xpf WT xpf
pGF - - + + + + +
pFP - + - + + + +

Figure 2

for removal of short non-homologous ends during SSA recombination *in planta*, and bring to light an unexpected inhibitory role for XPF on homologous recombination in this context.

The XPF-independence of SSA recombination in Arabidopsis involving short non-homologous tails was further confirmed by monitoring inter-molecular recombination between linearized plasmids co-transfected into protoplasts from WT and *xpf* mutant plants (**Figure 2)**. The pGF plasmid contains a 35S promoter followed by 3' truncated GFP gene encoding the N-terminal region of the GFP protein. The pFP plasmid contains the 5' truncated GFP gene encoding the C-terminal region of the GFP protein, and the nopaline synthetase (NOS) terminator. The overlapping common homology region of the truncated GFP genes in the two plasmids is 607 bp long. Linearization of pGF and pFP with HpaI and SnaBI respectively test SSA recombination in the absence of non-homologous ends ("No tail"). Digestion of pGF and pFP by SnaBI and HpaI (respectively) generates short non-homologous tails (of 13 and 10 nucleotides, respectively) which must be removed for completion of recombination between the two genes (**Figure 2A**). The digested plasmids were co-transfected into WT and *xpf* mutant Arabidopsis protoplasts and GFP+ cells quantified by flow cytometry (Materials and Methods). As shown in **Figure 2B**, frequencies of SSA events are equivalent in the two lines in the presence or absence of short non-homologous tails. XPF is thus not required for removal of short non-homologous tails (< 13 nucleotides) during SSA processing in Arabidopsis cells.

XPF inhibits SDSA recombination

DGU.US -> GUS recombination is thus more efficient in *xpf* mutant than in WT plants. Given that the absence of RAD51 does not reduce the efficiency of DGU.US -> GUS recombination [51,52], productive (GUS+) recombination at the DGU.US locus occurs majoritarily through RAD51-independent SSA. RAD51-dependent recombination can

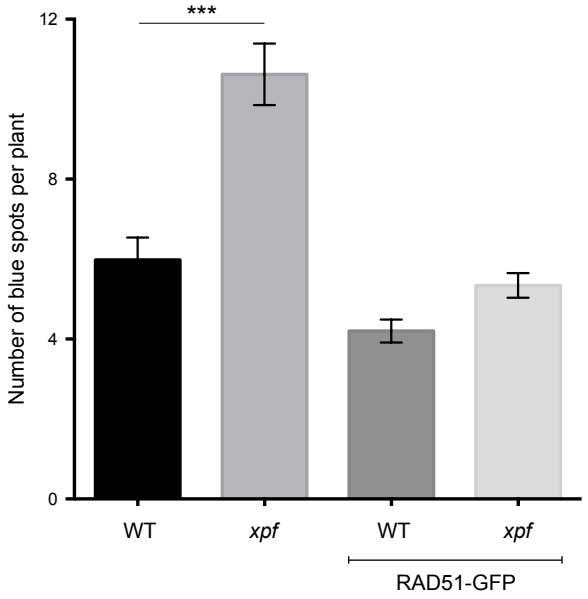

Figure 3

however also yield GUS+ [53,54] and it was thus necessary to clarify the question of the relative contributions of RAD51-dependent (SDSA/GC) and -independent (SSA) recombination in the hyper-recombination phenotype of the *xpf* mutant plants. To test this we knocked-out RAD51 activity in the DGU.US lines by expressing the dominant-negative RAD51-GFP fusion protein [55]. Knocking-out RAD51 strand-exchange activity almost completely eliminates the hyper-recombination phenotype of *xpf* mutant plants, while having little effect on spontaneous DGU.US recombination in control WT plants as expected (**Figure 3**). RAD51-dependent SDSA/GC thus accounts for the observed increase in spontaneous DGU.US -> GUS recombination in the absence of XPF.

To confirm and extend this conclusion, the analysis was carried out with a different recombination tester locus which specifically scores RAD51-dependent SDSA/GC recombination. Productive (GUS+) recombination at the IU.GUS locus involves the use of an internal fragment of the GUS gene (5' and 3' deleted) as donor sequence to repair DSB in an interrupted (by an inserted I-SceI site), but otherwise complete GUS gene [50]. Repair of an I-SceI induced DSB at the IU.GUS involves the removal of two short (16 and 18 nt) non-homologous tails and would thus be expected to give similar results to those observed for the DGU.US locus (above).

Spontaneous SDSA/GC recombination at IU.GUS was monitored in WT and *xpf* mutant plants (**Figure 4B**) and I-SceI induced SDSA recombination was tested in plants carrying the I-SceI expression cassette (homozygotes) and grown in the presence of the inductor β-estradiol (**Figure 4C**). As seen for the DGU.US -> GUS recombination, these analyses show a significant increase (more than 2-fold) of SDSA/GC events in *xpf* mutants compared to WT plants (**Figure 4)**. This increase is observed for both spontaneous and DSB-induced recombination. Control experiments with the double *xpf xrcc2* mutant confirmed the dependence of this effect on the RAD51 paralogue XRCC2, which we have previously shown

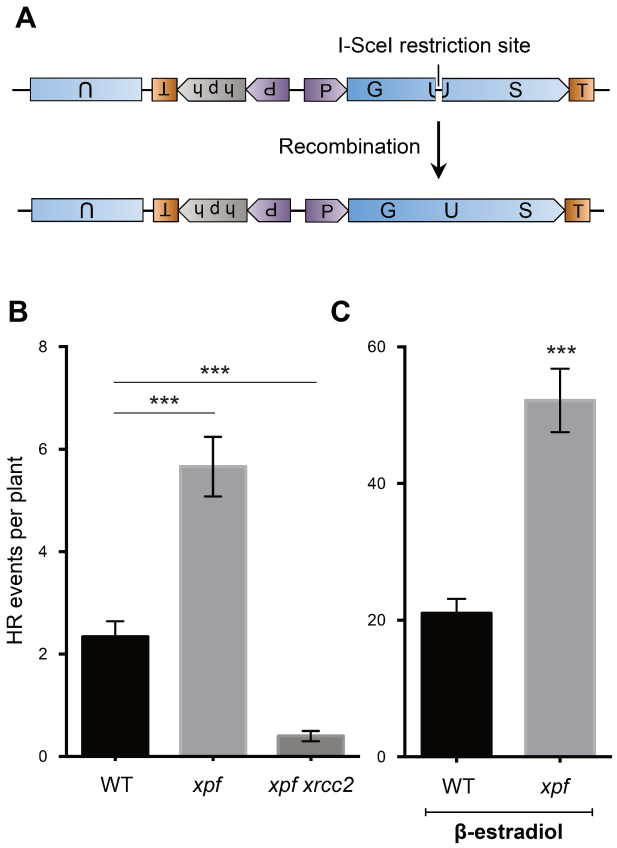
A
I-SceI restriction site
Recombination
B
C
HR events per plant
***
***
WT
xpf
xpf xrcc2
WT
xpf
β-estradiol

Figure 4

to be required for SDSA and SSA recombination in Arabidopsis [52]. Given that the structure of this locus precludes productive GUS+ recombination through RAD51-independent SSA, the increased recombination observed in *xpf* mutant plants results from RAD51-dependent SDSA/GC homologous recombination. An independent repetition of this experiment confirmed these results (**Table 2**) and as expected a similar effect was observed in *ercc1* mutant plants (**Figure S1**). Taken together, these data clearly demonstrate an inhibitory action of the XPF-ERCC1 complex on SDSA recombination.

The nuclease activity of XPF is required for inhibition of SDSA

The catalytic activity of the XPF-ERCC1 site-specific endonuclease is carried on the XPF subunit [19,20]. The amino acids essential for the catalytic activity have been identified in the human protein using site-directed mutagenesis and are highly conserved among eukaryotes [20]. To determine whether the inhibition of SDSA by XPF-ERCC1 is due to its endonuclease activity or to some other role of the complex, we constructed nuclease-minus (and WT) XPF-GFP translational fusions driven by the XPF promoter region (**Supplementary Figures 2A and 3A**; Materials and Methods). The nuclease-minus XPF protein, $XPF_{D757A,}$ carries the equivalent amino acid substitution which in the human protein disrupts XPF catalytic activity but has no influence in DNA binding activity [20]. XPF-GFP and XPF_{D757A}-GFP were introduced independently into *xpf* mutant plants, transformants selected and expression of the fusion protein confirmed by visual examination of GFP fluorescence. The presence of XPF-GFP fully complements XPF-deficient plants for repair of UV- and MMC-induced DNA damage, while the XPF_{D757A}-GFP does not, confirming that XPF-GFP is active and functional in DNA repair, while the nuclease-minus XPF_{D757A}-GFP protein is inactive (**Supplementary Figures 2 and 3**).

We introduced the IU.GUS recombination reporter locus into these lines and

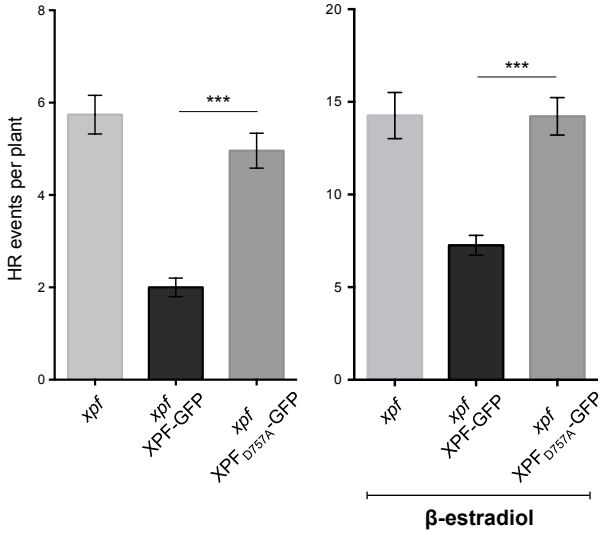
HR events per plant
8
6
4
2
0
***
xpf
xpf XPF-GFP
xpf XPF$_{D757A}$-GFP
20
15
10
5
0
***
xpf
xpf XPF-GFP
xpf XPF$_{D757A}$-GFP
β-estradiol

Figure 5

monitored spontaneous SDSA recombination at IU.GUS in *xpf* XPF-GFP and *xpf* XPF_{D757A}-GFP plants (**Figure 5**). As expected, complementation of *xpf* mutants with the XPF-GFP fusion protein gives SDSA recombination rates equivalent to WT plants, however this is not the case with the nuclease-minus XPF_{D757A}-GFP expressing line, which has a similar phenotype to the *xpf* mutant. The inhibitory role of XPF on SDSA recombination in Arabidopsis is thus due to its nuclease activity.

DISCUSSION

In addition to its well-characterized role in NER, the role of the XPF-ERCC1 complex in homology-directed DSB repair is well established (for reviews, see [56,57]) and has been shown to be due to cleavage of non-homologous 3' tails of recombination intermediates produced by both Single Strand Annealing and Synthesis Dependent Strand Annealing pathways [17,25-31]. The need for this activity however depends on both the length of the non-homologous tails, and their number (reviewed by [35]). The results presented here confirm that, as is the case in yeast and in animals, this requirement for Arabidopsis XPF-ERCC1 endonuclease in both SSA and SDSA pathways depends on the length of non-homologous 3' tails: the complex is needed to cleave long non-homologous 3' tails but is dispensable for the removal of short tails (< 21 nucleotides) *in planta* (this work and [28,29]).

Using a specific reporter for SDSA recombination (the IU.GUS substrate), we show that recombination efficiency is significantly increased (more than 2-fold) in *xpf* mutants relative to the WT controls (**Figure 4, Table 2**). This effect is seen in both spontaneous and I-SceI induced recombination (**Figure 4, Table 2**) and as expected, absence of ERCC1 shows the same effect (**Figure S1**). Complementation of *xpf* mutant plants with the XPF-GFP protein confirms that the increase of SDSA efficiency is specifically due to the absence of XPF (**Figure 5**). In contrast to the control XPF-GFP fusion protein, the presence of XPF_{D757A}-GFP has no effect on SDSA recombination rate in *xpf* mutant plants (**Figure 5**), confirming that the inhibitory role of XPF on SDSA is dependent on its ability to cleave DNA. A similar hyper-recombination effect of the absence of XPF is seen with the DGU.US recombination tester locus (**Figure 1, Table 1**). DGU.US -> GUS recombination can in principle occur through SSA and SDSA recombination [54], although its independence of RAD51 strongly suggests that it occurs principally *via* SSA [51,52]. The increased recombination in *xpf* mutant

plants is however fully RAD51-dependent, as seen in the wild-type phenotype of *xpf* plants lacking RAD51 strand-exchange activity (expressing the dominant-negative RAD51-GFP) (**Figure 3**).

Our results thus show increased numbers of recombinant GUS+ spots in the absence of XPF endonuclease activity. This effect is observed in both the DGU.US and IU.GUS recombination tester loci and thus concerns both SDSA (IU.GUS and possibly DGU.US) and SSA (DGU.US) recombination. In both cases this increase is RAD51-dependent and thus absence of XPF results in an additional pool of SDSA events at these loci. It is possible that this reflects increased initiation of recombination (eg DSB) in *xpf* mutants, however the fact that *xpf* mutants are not general hyper-rec. and that we see similar effects on spontaneous and I-SceI induced recombination would argue against this. Taking this reasoning further, there must exist a pool of events which are repaired by non-productive (-> gus-) recombination in the presence of XPF, and by productive SDSA (-> GUS+) recombination in *xpf* mutants.

Numerous studies in yeasts, animals and plants have reported that repair of DSB arising between tandem direct repeated sequences mostly results in deletion events ascribed to the SSA pathway [32,33,50,58,59]. In *S. cerevisiae*, SSA is nearly 100% efficient when homologous regions flanking the DSB are at least 400 bp long [60]. Decreasing length of the repeated sequences and increasing sequence divergence both reduce the efficiency of SSA (3 % divergence between 205 bp repeats reduced SSA by a factor of 5) - as does to a lesser extent increased distance between repeats [36,59,60]. Similarly, introduction of DSB into one of two chromosomal repeats in hamster cells leads more frequently to deletion events than GC events (approximately 3:1 ratio) [33]. Consistent with these results, Orel and colleagues showed that recombination associated with deletions is the most efficient pathway of homologous DSB repair in Arabidopsis [50].

A model based on homology search competition has been proposed to explain the

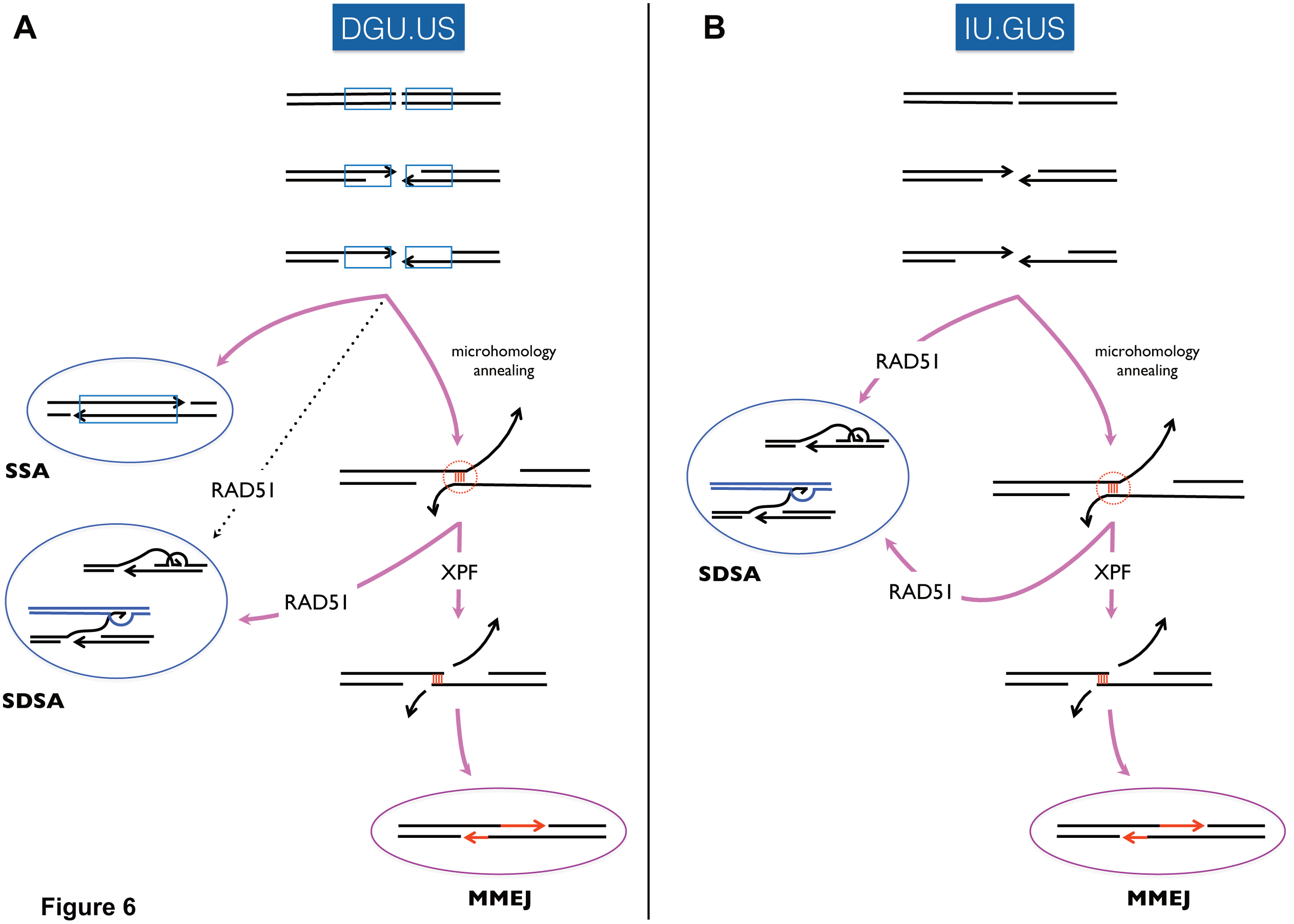
A
DGU.US
microhomology
annealing
SSA
RAD51
RAD51
XPF
SDSA
MMEJ
B
IU.GUS
RAD51
microhomology
annealing
SDSA
RAD51
XPF
MMEJ

Figure 6

preference for intra-chromosomal donors for homologous repair [59]. Following formation of the DSB and concomitantly with resection of the DSB ends, base pairing is examined in order to find homology between the resected strands (*in cis*), while the ends search for inter- or intra-chromosomal homology (*in trans*). Homology search *in cis* and *in trans* are simultaneous, and the choice of repair pathway depends on the first region of homology identified. According to this model, base pairing during homology search *in cis* generates ssDNA/dsDNA junctions, substrates of Rad1-Rad10 (XPF-ERCC1) endonuclease. The cleavage of 3' single strand tails by the complex would thus limit homology search *in trans* and, in turn, SDSA/GC recombination. XPF-ERCC1 would thus promote deletion-associated repair by inhibiting SDSA/GC recombination and favouring SSA.

In Figure 6 we present a model to explain our results based on this action of XPF-ERCC1 in limiting homology search through cleavage of a substrate generated by annealing of microhomologies during resection. By analogy with the model presented above [59], we propose that the XPF-ERCC1 complex recognizes the ssDNA/dsDNA junctions formed by annealing of microhomologies (during homology search *in cis*) and cleaves the 3' single strand tails (**Figure 6**). XPF-ERCC1 cleavage would thus disfavour homology search *in trans* and result in non-productive microhomology-mediated end-joining (MMEJ) events (gus-). In the absence of XPF-ERCC1 endonuclease activity, the enhanced stability of long 3' single-strand ends would promote homology search *in trans*, hence favouring SDSA/GC recombination (**Figure 4, Table 2, Figure S1**). Thus, we propose that the observed hyper-recombination phenotype in the absence of XPF nuclease activity is the result of competition between homology research *in cis* and *trans*.

MATERIALS AND METHODS

Plant material and growth conditions

The *Arabidopsis thaliana xpf* (uvh1-1 (Harlow *et al.*, 1994), *ercc1* (Dubest *et al.*, 2004) and *xrcc2* (Bleuyard *et al.*, 2005) mutants used in this work have been described previously. Single and double mutants were crossed with the recombination tester DGU.US-1 or IU.GUS-8 lines (Orel *et al.*, 2003) and mutants homozygous for the DGU.US or IU.GUS substrate loci were identified in the F2, then used for spontaneous recombination tests. Wild-type lines homozygous for DGU.US or IU.GUS substrates were transformed with the inducible I-SceI expression cassette previously described (I-SceI coding sequence under control of β-estradiol) (Serra *et al.*, 2013) utilising the floral dip method (Clough & Bent, 1998). T2 plants homozygotes for the I-SceI expression cassette (and DGU.US or IU.GUS substrate) were subsequently crossed with the mutant lines homozygous for DGU.US or IU.GUS substrate previously generated. F2 homozygotes for each construct were used for I-SceI induced recombination tests. Wild-type control plants come from the same crosses.

Plants were grown under standard conditions: surface-sterilized seeds were stratified at 4°C for 2 days and grown *in vitro* on germination medium (0.8% w/v agar, 1% w/v sucrose and half-strength Murashige & Skoog salts (M0255; Duchefa Biochemie, Netherlands)) in a growth cabinet with a 16-h light/8-h dark cycle, at 23°C with 45–60% relative humidity. The growth medium was supplemented with 170 μM 17-β-estradiol (E2758; Sigma-Aldrich) for induction of I-SceI expression.

Plasmid constructs

As control for transformation efficiency, we used the pGFP plasmid containing the cauliflower mosaic virus (CaMV) 35S promoter, the GFP gene and the nopaline synthetase

(NOS) transcription terminator. The 35S promoter sequence and the GFP open reading frame without the last 49 bp were amplified from pGFP by PCR (forward primer GATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTC and reverse primer TACGTATCGCGAAGTTAACTCCAGCAGGACC) and inserted into the pGEM-T Easy (Promega) to produce the pGF plasmid. The GFP open reading frame without the first 64 bp and the NOS terminator was amplified from pGFP by PCR (forward primer GTTAACTCGCGAATACGTAAACGGCCACAAGTTCAG and reverse primer AGCGCCCAATACGCAAACCGCCTCTCCCC) and inserted as above into the pGEM-T Easy to produce the pFP plasmid. Both generated plasmids were verified by sequencing.

pGF and pFP plasmids were linearized with *Hpa*I or *Sna*BI, respectively, to prepare the "No tail" recombination substrate. For the "With short tails" recombination substrate, pGF and pFP plasmids were digested by *Sna*BI and *Hpa*I, respectively. In both cases, the overlapping homology region is 607 bp to which pGF/*Sna*BI adds a non-homologous tail of 13 nucleotides and pFP/*Hpa*I a tail of 10 nucleotides. Digested plasmids were purified and concentrated with QiaexII kit (Qiagen) as recommended by the manufacturer. DNA concentrations were adjusted to 1 µg/µl before protoplast transfection.

Protoplast isolation and transfection

The Arabidopsis protoplasts were isolated based on the methods reported by Sheen laboratory (Yoo *et al.*, 2007) with minor modifications. Briefly, leaves from 3-4 week old WT or *xpf* mutant plants were cut into 0.5-1 mm wide strips with fresh razor blades. The leaf strips were quickly transferred into 8 ml of digestion solution (0.25% w/v Macerozyme Onozuka R-10, 1% w/v Cellulase Onozuka R-10 (Yakult, Japan), 0.4 M mannitol, 20 mM 4-morpholineethanesulfonic acid (MES) pH 5.7, 20 mM KCl, 10 mM $CaCl_2$, 5 mM β-mercaptoethanol and 0.1% bovine serum albumin (BSA)) and vacuum applied for 30 min.

The vacuum was released gently and samples incubated for 3-h without shaking in the dark at 23°C. After incubation, the digestion solution was filtered through 30 µm nylon mesh to eliminate leaf debris. The Petri dish was rinsed with 8 ml of W5 solution (125 mM $CaCl_2$, 154 mM NaCl, 5 mM KCl, 2 mM MES pH 5.7) with shaking to release the remaining protoplasts followed by filtration. The pooled protoplast suspensions were then centrifuged at 100g for 2 min. The collected protoplasts were gently washed once with cold W5 solution and incubated 30 min on ice in 2 ml of W5 solution. The protoplast solution was subsequently centrifuged and the pellet resuspended in room temperature MaMg solution (0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES pH 5.7). Protoplast concentrations were quantified by microscopy using Malassez cytometer slide and the protoplast concentration adjusted to 1.5 x 10^6/ml.

All steps of protoplast transfection were carried out at 23°C. 100 µl (1.5 x 10^5) of protoplasts were gently mixed with 10 µl (10 µg) of each plasmid (digested pGF and pFP) and then 110 µl of 40% v/v polyethylene glycol solution (PEG-4000 (Fluka #81240), 0.8M mannitol, 1M $CaCl_2$ and 1M KCl). The transfection mixture was incubated for 30 min, then diluted in 15 ml of W5 solution and centrifuged at 100g for 2 min. The protoplasts were gently resuspended in 1 ml of WI solution (0.5 M mannitol, 0.5 M KCl and 4 mM MES pH 5.7) and then incubated for 24-h at 23°C in the dark without shaking. For each preparation, 100µl of WT and *xpf* mutant protoplasts were transfected in parallel with 10µl (10 µg) of pGFP to quantify the transfection efficiency in each transformation experiment.

GFP fluorescence analyses by flow cytometer

Transfected protoplasts were filtered through 30 µm nylon mesh and analysed directly in WI solution. Flow cytometric analyses were performed with an Attune Acoustic Focusing Cytometer (Life Technologies). GFP fluorescence excited with a 488 nm diode laser was detected using a 530/30 nm bandpass filter in the BL1 channel.

Histochemical staining of GUS expression

Fourteen-day old seedlings grown under standard conditions (supplemented or not with 17-β-estradiol) were harvested and incubated in staining buffer (0.2% Triton X-100, 50 mM sodium phosphate buffer (pH 7.2), 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; Biosynth), dissolved in N,N-dimethylformamide). Plants were infiltrated under vacuum for 15 min and incubated at 37°C overnight. The staining solution was then replaced with 70% ethanol to remove leaf pigments and the blue spots were counted under a binocular microscope.

Cloning of XPF, mutagenesis and plant transformation

To analyse the role of the XPF nuclease activity, the genomic region without stop codon (3873 bp) and a 1410 bp 5' upstream sequence of XPF was amplified (forward primer TGATTAGCATTTAGCGTCAAG and reverse primer ATCCTTGCAATCTGTTACACC), inserted into pDONR221 and verified by sequencing. This pDONR221_pXPF::XPF_{wt} was then used as a template for site-directed mutagenesis. Mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's instructions. To generate a plasmid carrying the a3089c mutation in the XPF active site, equivalent to that described by Enzlin and Schärer (2002), two oligonucleotide primers complementary to opposite strands of the target sequence and both containing the mutation (forward primer TACACTAGAGGTCGGCG**c**CTATATTCTATCTCCTTC and reverse primer GAAGGAGATAGAATATAG**g**CGCCGACCTCTAGTGTA) were synthesized and extended by thermal cycling using *PfuUltra* HF DNA polymerase. This generates the pDONR221_pXPF::XPF_{D757A} carrying the a3089c mutation in the XPF genomic sequence. The reaction was then treated with *Dpn*I enzyme to digest methylated and hemimethylated parental plasmid DNA. The remaining extended plasmid DNA containing the

a3089c mutation was subsequently transformed into XL10-Gold ultracompetent cells. The pDONR221_pXPF::XPF_{wt} and the pDONR221_pXPF::XPF_{D757A} were verified by sequencing and cloned into the GATEWAY destination vector pB7FWG2 from which the 35S promoter had been removed with a *Sac*I/*Spe*I digest (Karimi *et al.*, 2007). This generates a translational fusion of eGFP to the C-terminus of the XPF. The pXPF-GFP and pXPF_{D757A}-GFP plasmids were transformed into the *Agrobaterium tumefaciens* C58C1 strain and used to transform *xpf* mutant plants (Harlow *et al.*, 1994) by the floral dip method (Clough & Bent, 1998).

GFP fluorescence observation of XPF-GFP fusion proteins

Expression of the XPF-GFP and XPF_{D757A}-GFP fusion proteins in transformed plants was analysed by observation of GFP fluorescence in roots of five day-old seedlings using a motorised Zeiss AxioImager.Z1 epifluorescence microscope (Carl Zeiss AG, Germany). Photographs were taken with an AxioCam Mrm camera and Zeiss filter set 38HE. Images were further treated using the AxioVision 4.6.2 software (Carl Zeiss AG, Germany).

UV-light and Mitomycin C sensitivity assays

For UV sensitivity assays, seeds were surface-sterilised and germinated on vertical plates containing 0.8% w/v agar, 1% w/v sucrose and half-strength Murashige and Skoog salts medium. Five-day-old seedlings were then treated with 300 $J.m^{-2}$ UV-C radiation (GS Gene linker; Bio-Rad, Hercules, CA, USA), allowed to grow for 5 days in the dark to avoid photoreactivation and final root length was measured.

For the Mitomycin C sensitivity assay, seeds were sown on plates containing fresh solid germination medium supplemented with 40 µM Mitomycin C (M0503; Sigma-Aldrich). The plates were then incubated for 17 days (16-h light, 23°C) and growth/death of plantlets scored visually.

FIGURE LEGENDS

Figure 1. Spontaneous and I-SceI induced DGU.US recombination is increased in the *xpf* mutant.

(A) Schematic map of the DGU.US chromosomal recombination reporter locus.

(B) Examples of WT and *xpf* mutant plants after GUS staining. Scale bar = 2 mm.

(C) Counting shows that *xpf* mutant plants have significantly more blue recombinant GUS+ spots than wild-type controls. Means ± standard errors of the means. *** $p < 0.0001$ (Mann-Whitney test) N= 50

(D) The increased numbers of recombinant GUS+ spots are also seen after induction of GUS recombination by I-SceI cleavage using a β-estradiol-inducible I-SceI expression cassette. Means ± standard errors of the means. *** $p < 0.0001$ (Mann-Whitney test) N= 50

Figure 2. XPF is not required for the removal of short non-homologous tails during SSA recombination.

(A) Recombination assay. Linear recombination substrates were prepared from pGF and pFP plasmids by restriction with enzymes that leave (with tails) or not (no tail) non-homologous DNA overhangs. The "no tail" test was carried out with pGF/*Hpa*I and pFP/*Sna*BI and the "with short tails" test with pGF/*Sna*BI and pFP/*Hpa*I. The short tails are 13 and 10 nucleotides respectively. Resection of the linear molecules permits annealing of the exposed complementary sequences. The annealing of the 3'-ended strands from "with short tails" plasmids generates unpaired overhangs, which must be removed to permit re-synthesis and strand ligation to restore a complete functional GFP gene.

(B) The linearized plasmids were co-transfected into WT and *xpf* mutant protoplasts either alone or together and restoration of the GFP gene was scored by flow cytometry 24h after transfection. The relative numbers of WT and *xpf* mutant GFP+ protoplasts are presented

relative to the transfection with a circular GFP containing plasmid (100%). Median with range, N > 1800 protoplasts, 2 repeats.

Figure 3. The increase of spontaneous DGU.US recombination in *xpf* mutant plants depends upon RAD51.

The significant increase in spontaneous recombination rate in *xpf* mutant plants is absent in the presence of the dominant-negative RAD51-GFP. Mean numbers of recombinant GUS+ spots per plant ± standard errors of the means. *** $p < 0.0001$ (Mann-Whitney test). N=50

Figure 4. Spontaneous and I-SceI induced IU.GUS recombination is increased in the *xpf* mutant.

(A) Schematic map of the IU.GUS chromosomal recombination reporter locus. Both spontaneous (B) and I-SceI-induced (C) IU.GUS recombination rates are significantly increased in *xpf* mutant plants. Absence of XRCC2 completely eliminates the *xpf* hyper-rec phenotype, confirming that it is the result of increased homologous recombination. Mean numbers of recombinant GUS+ spots per plant ± standard errors of the means. *** $p < 0.0001$ (Mann-Whitney test).

Figure 5. XFP-GFP but not XPF_{D757A}-GFP restores the wild-type IU.GUS recombination rate in the *xpf* mutant.

Spontaneous IU.GUS -> GUS recombination was monitored in two F1 populations from independent crosses for each genotype. *xpf* mutant plants expressing the nuclease-minus XPF_{D757A}-GFP retain the hyper-rec phenotype of the *xpf* mutant, while those complemented by the wild-type XPF-GFP fusion protein are not hyper-rec. *xpf/xpf* plants are hemizygous for the IU.GUS locus and the XPF- GFP/XPF_{D757A}-GFP construct. Mean numbers of recombinant GUS+ spots per plant ± standard errors of the means. *** $p < 0.0001$ (Mann-Whitney test).

Figure 6. A competition model to explain the increased homologous recombination in the *xpf* mutant.

By analogy with the model proposed by Agmon et al (2009), we propose that the hyper-rec phenotype of *xpf* mutants is due to shifting of the balance between productive HR and non-productive MMEJ recombination pathways.

(A) The tandem direct repeat DGU.US locus can generate a functional GUS gene *via* SSA or SDSA recombination. (B) To generate a functional GUS gene from the IU.GUS substrate, the interrupted GUS copy must recombine with the 5' and 3' deleted donor through RAD51-dependent recombination (SDSA). (A and B) Following I-SceI induced DSB formation, resection of both sides of the break generates 3'-ended ssDNA which will participate in the search for homology. Concomitantly with the resection, exposed complementary microhomologies can anneal and these present a substrate for the XPF-ERCC1 endonuclease. Cleavage of the ssDNA tails of the microhomology-annealing intermediate by XPF-ERCC1 would block the search for homology, and repair of the DSB through microhomology-mediated end-joining (MMEJ) will not generate a functional GUS gene. In the absence of XPF or ERCC1, these long 3' single-strand ends would promote homology search *in trans*, thus favouring RAD51-dependent SDSA recombination and producing the observed increase in RAD51-dependent recombination in *xpf* mutant plants.

SUPPORTING INFORMATION LEGENDS

Figure S1. I-SceI induced IU.GUS recombination is increased in the *ercc1* mutant.

ercc1 mutant plants have significantly more blue recombinant GUS+ spots than wild-type controls after induction of GUS recombination by I-SceI cleavage using a β-estradiol-inducible I-SceI expression cassette. Means ± standard errors of the means. *** $p < 0.0001$ (Mann-Whitney test). $N \geq 28$

Figure S2. XPF-GFP restores UV-resistance of the *xpf* mutant plants.

(A) Schematic representation of the XPF-GFP fusion construct.

(B-C) Sensitivity of *xpf* plants expressing or not XPF-GFP to DNA damage induced by ultraviolet (UV) radiation. Six T3 lines from *xpf* plants transformed with XPF-GFP construct were analysed. Five day-old seedlings were treated with 300 $J.m^{-2}$ UV radiations, incubated for 5 days in the dark and length of the roots measured. Mean root lengths ± standard errors of the means.

Figure S3. XPF_{D757A}-GFP does not restore UV- and MMC resistance of the *xpf* mutant plants.

(A) Schematic representation of the XPF_{D757A}-GFP fusion construct. The adenine 3089 of the XPF genomic sequence was replaced by cytosine using site-directed mutagenesis (Materials and Methods). This mutation induces the substitution of the aspartic acid to alanine at position 757 of the XPF protein.

(B) XPF_{D757A}-GFP is expressed in nuclei of root meristematic cells. Differential interference contrast (DIC) + GFP (upper panel) and GFP fluorescence (lower panel) images are shown. Scale bar = 50 μm.

(C) Sensitivity of *xpf* plants expressing or not XPF_{D75A}-GFP to DNA damage induced by UV radiation. Four T3 lines from *xpf* plants transformed with XPF_{D757A}-GFP construct and expressing the fusion protein were analysed. Five day-old seedlings were treated with 300 $J.m^{-2}$ UV radiations, incubated for 5 days in the dark and length of the roots measured. Mean root lengths ± standard errors of the means.

(D) Sensitivity of *xpf* plants expressing XFP-GFP or XPF_{D757A}-GFP to DNA crosslinking agent Mitomycin C (MMC). Expression of the XPF-GFP protein restores MMC resistance to *xpf* plants, while the XPF_{D757A}-GFP does not. Seventeen day-old seedlings grown in presence of 40 μM of MMC are shown.

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Table 1. Spontaneous and I-SceI induced DGU.US recombination in *xpf* mutant and in wild-type plants.

		Spontaneous DGU.US recombination				***I-SceI induced DGU.US recombination***			
Experiment		*n*	*N*	*m ± SEM*	*Ratio*	*n*	*N*	*m ± SEM*	*Ratio*
1	**WT**	49	293	5.98 ± 0.56		50	941	18.82 ± 1.63	
	xpf	50	531	10.62 ± 0.77	**1.776*****	50	1882	37.64 ± 2.95	**2.000*****
2	**WT**	50	412	8.24 ± 0.50		50	2102	42.04 ± 2.77	
	xpf	50	601	12.02 ± 0.60	**1.459*****	51	2766	54.24 ± 3.24	**1.291****

Recombination in the mutants and wild-type was compared using non-parametric statistical analysis (Mann-Whitney test). Differences between *xpf* and wild-type are highly significant in all cases (** $p < 0.005$; ***$p < 0.0001$). n, the number of plantlets screened; N, the total number of blue spots (recombination events); m ± SEM, the mean number of recombination events per plant ± standard error of the mean.

Table 2. Spontaneous and I-SceI induced IU.GUS recombination in *xpf* mutant and in wild-type plants.

		Spontaneous IU.GUS recombination				***I-SceI induced IU.GUS recombination***			
Experiment		*n*	*N*	*m ± SEM*	*Ratio*	*n*	*N*	*m ± SEM*	*Ratio*
1	**WT**	50	117	2.34 ± 0.30		50	1050	21.00 ± 2.12	
	xpf	50	283	5.66 ± 0.58	**2.419**	50	2608	52.16 ± 4.64	**2.484**
	xpf xrcc2	50	20	0.40 ± 0.10	**0.171**				
2	**WT**	50	180	3.60 ± 0.36		49	1159	23.65 ± 3.27	
	xpf	50	373	7.46 ± 0.88	**2.072**	51	2923	57.31 ± 4.52	**2.423**
	xpf xrcc2	50	27	0.54 ± 0.12	**0.150**				

Recombination in the mutants and wild-type were compared using non-parametric statistical analysis (Mann-Whitney test). Differences between *xpf* and wild-type are highly significant in all cases ($p < 0.0001$). n, the number of plantlets screened; N, the total number of blue spots (recombination events); m ± SEM, the mean number of recombination events per plant ± standard error of the mean.

Table 3. Spontaneous and I-SceI induced IU.GUS recombination in *xpf* mutants expressing or not XPF-GFP or XPF_{D757A}-GFP.

		Spontaneous IU.GUS recombination			***I-SceI induced IU.GUS recombination***		
Experiment		*n*	*N*	*m ± SEM*	*n*	*N*	*m ± SEM*
1	***xpf***	50	287	5.74 ± 0.42	50	713	14.26 ± 1.24
	xpf XPF-GFP	50	100	2.00 ± 0.20	50	363	7.26 ± 0.54
	xpf XPF_{D757A}***-GFP***	50	248	4.96 ± 0.38	50	711	14.22 ± 1.01
2	***xpf***	50	339	6.78 ± 0.44	54	554	10.26 ± 0.55
	xpf XPF-GFP	50	149	2.98 ± 0.21	53	319	6.02 ± 0.46
	xpf XPF_{D757A}***-GFP***	50	283	5.66 ± 0.38	53	595	11.23 ± 0.94

n, the number of plantlets screened; N, the total number of blue spots (recombination events); m ± SEM, the mean number of recombination events per plant ± standard error of the mean.

Figure S1

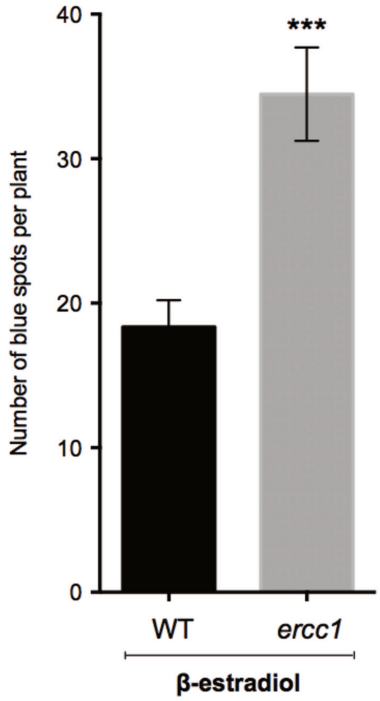

40
30
20
10
0
***
Number of blue spots per plant
WT
ercc1
β-estradiol

Figure S2

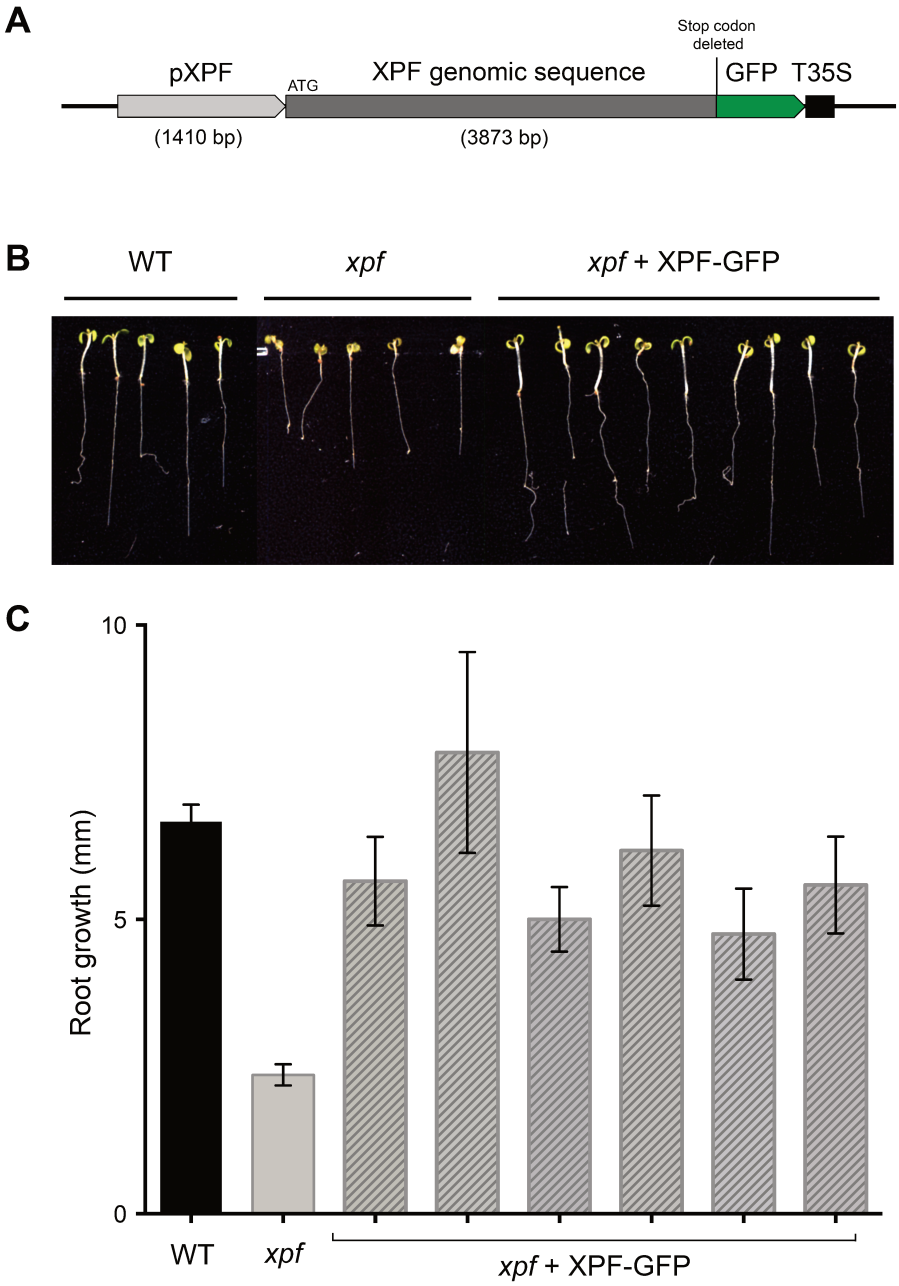
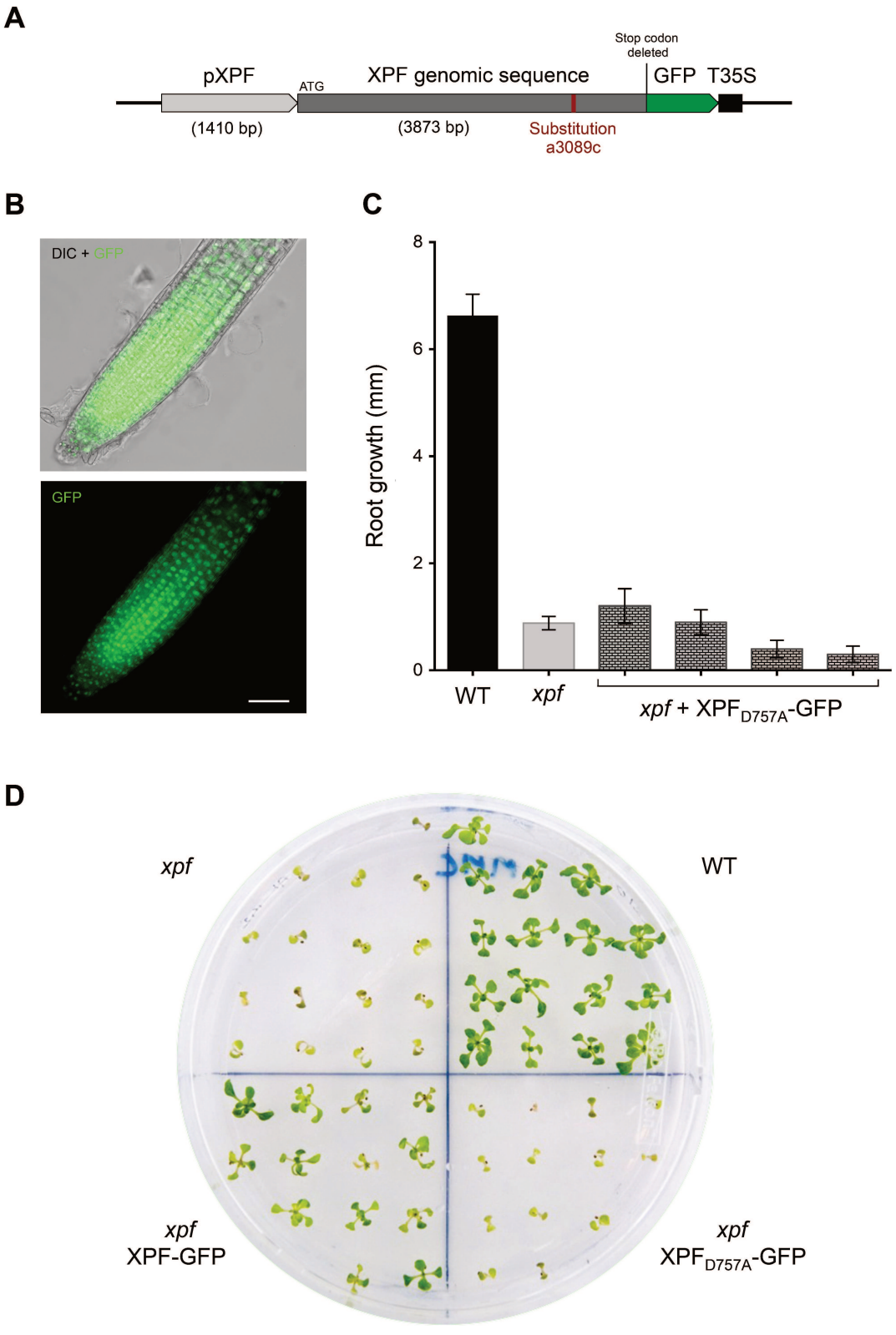

A
Stop codon deleted
pXPF
ATG
XPF genomic sequence
GFP
T35S
(1410 bp)
(3873 bp)
B
WT
xpf
xpf + XPF-GFP
C
Root growth (mm)
10
5
0
WT
xpf
xpf + XPF-GFP

Figure S3


A
Stop codon deleted
pXPF
ATG
XPF genomic sequence
GFP T35S
(1410 bp)
(3873 bp)
Substitution a3089c
B
DIC + GFP
GFP
C
Root growth (mm)
8
6
4
2
0
WT
xpf
xpf + XPFD757A-GFP
D
xpf
WT
xpf
XPF-GFP
xpf
XPFD757A-GFP

Chapitre III

Mise au point d'un système rapporteur d'évènements de jonction d'extrémités d'ADN en protoplastes d'*Arabidopsis thaliana*

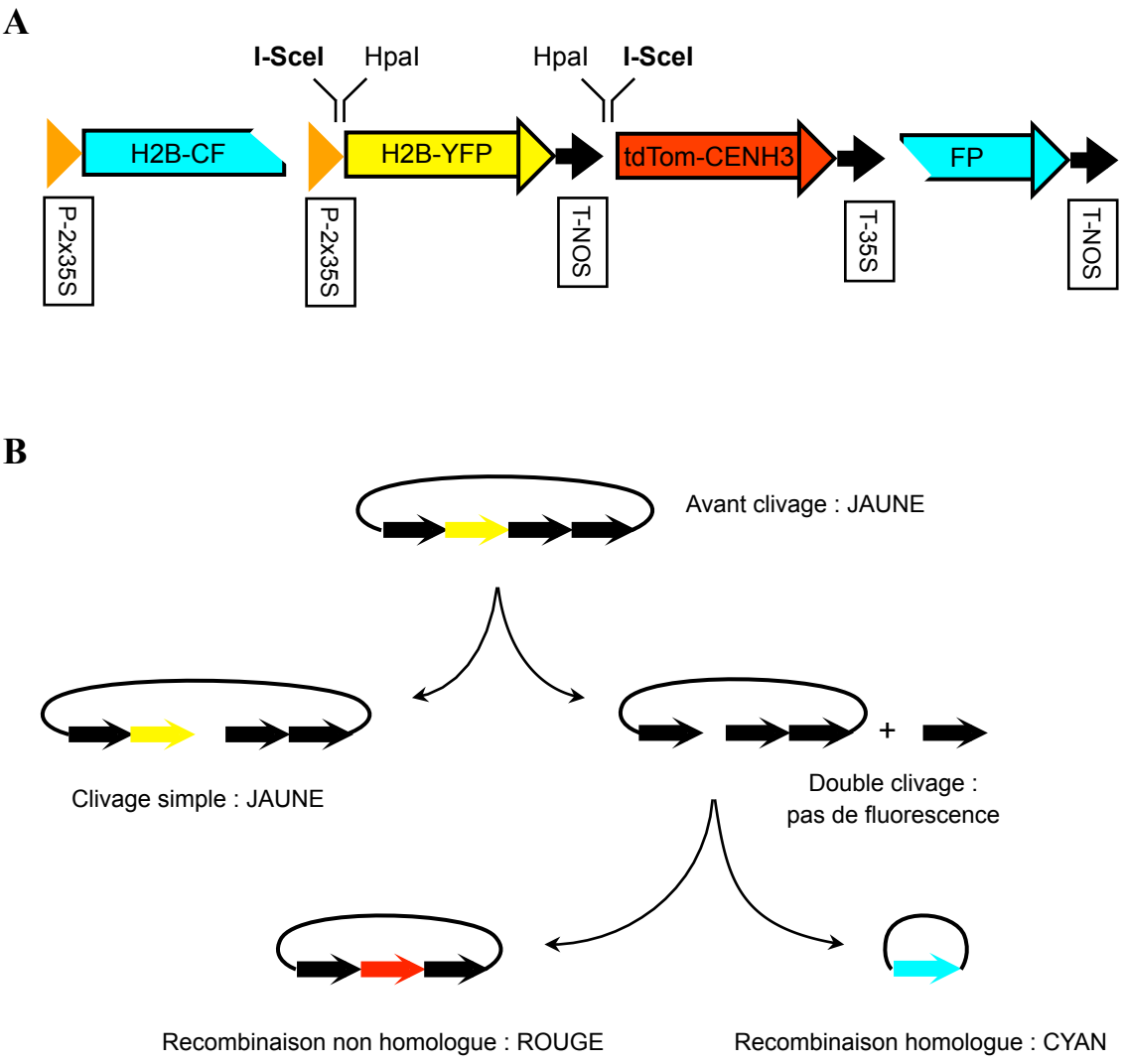


Figure 21 : Le plasmide rapporteur de recombinaison

(A) Carte schématique de la construction rapportrice de recombinaison. Elle est composée de deux copies tronquées du gène de fusion H2B-CFP (rectangles cyans) portant une séquence commune de 564 pb, et des gènes de fusion H2B-YFP (rectangle jaune) et tdTomato-CENH3 (rectangle rouge). Les triangles oranges représentent les promoteurs et les flèches noires, les terminateurs. P-2x35S : double promoteur 35S ; T-NOS : terminateur de la nopaline synthase ; T-35S : terminateur 35S ; I-SceI et HpaI : sites de restriction des enzymes I-SceI et HpaI, respectivement.

(B) Principe de la mise en évidence des évènements de recombinaison homologue et non homologue selon la couleur de la fluorescence (associée à la reconstitution d'un gène de fusion).

Afin d'étudier les interactions entre les voies de recombinaison, nous avons développé au laboratoire un système plasmidique permettant de distinguer les évènements de recombinaison non homologue des évènements de recombinaison homologue. Le plasmide initial a été construit par P. Fargier sur le même principe que les systèmes rapporteurs développés par plusieurs laboratoires, dont ceux de H. Puchta et B. Lopez (Salomon & Puchta, 1998 ; Siebert & Puchta, 2002 ; Guirouilh-Barbat *et al.*, 2004 ; Rass *et al.*, 2009). Notre système a pour but de quantifier *in vivo* l'efficacité de réparation de CDB générées par une enzyme de restriction, par les voies de jonction des extrémités d'ADN *vs* les voies de recombinaison homologue. Basé sur la reconstruction par recombinaison de gènes codant des protéines de fusion fluorescentes, ce système rapporteur peut être directement utilisé en protoplastes. Ceci simplifie considérablement les analyses dans différents contextes mutants, puisqu'il n'est pas nécessaire de réaliser des croisements avec chacune des lignées mutantes comme c'est le cas avec une construction chromosomique.

I. Présentation du plasmide rapporteur de recombinaison

Le plasmide rapporteur de recombinaison a été construit par clonage à partir de plusieurs gènes de fusion. Le choix s'est porté sur des protéines nucléaires afin de s'affranchir de la fragilité des protoplastes au moment de l'analyse. Ce plasmide est constitué de deux copies tronquées du gène de fusion H2B-CFP (séquence codante de l'histone H2B fusionnée avec le gène rapporteur CFP (Boisnard-Lorig *et al.*, 2001)) en orientation directe et présentant une séquence répétée de 564 pb (**Figure 21 A**). Entre ces régions, deux autres gènes de fusion ont été introduits : H2B-YFP sous le contrôle d'un double promoteur 35S (P-2x35S) et du terminateur de la nopaline synthétase (T-NOS) (Boisnard-Lorig *et al.*, 2001), suivi du gène tdTomato-CenH3 (séquence codante du gène rapporteur tdTomato fusionnée à celle de l'histone spécifique des centromères CENH3 (Kurihara *et al.*, 2008)) et du terminateur 35S.

Matériel

Feuilles de 1 à 2 cm de plantules d'environ 3 semaines cultivées sur terreau dans des conditions standards (16h jour/ 8h nuit, à 23°C avec une humidité relative de 45-60%).

Préparation des protoplastes

- Prélever une vingtaine de feuilles (sans le pétiole)
- Couper des bandes de 0,5 à 1 mm de largeur avec une lame de rasoir neuve dans une boîte de Pétri
- Ajouter 8 ml de solution de digestion et infiltrer sous vide pendant 30 min (à l'obscurité)
- Incuber 3h à 23°C à l'obscurité et sans agitation
- Agiter soigneusement la solution de digestion, la filtrer (filtre de 30 µm) et la recueillir dans un tube de 15 ml à bout arrondi
- Laver les fragments de feuilles avec environ 7 ml de W5 (à température ambiante) et filtrer la solution
- Centrifuger la solution de protoplastes à 100g pendant 2 min

Les protoplastes forment un culot vert bien visible

- Jeter le surnageant, resuspendre le culot dans 10 ml de solution W5
- Centrifuger à 100g pendant 2 min
- Jeter le surnageant, resuspendre le culot dans 2 ml de solution W5 et incuber 30 min sur de la glace
- Centrifuger à 100g pendant 2 min
- Jeter le surnageant, resuspendre le culot dans une solution MaMg
- Quantifier la concentration des protoplastes au microscope grâce à une cellule de Malassez et l'ajuster à 1,5 x 10^6/ml avec la solution MaMg

Transfection des protoplastes

Toutes les étapes sont effectuées à 23°C.

- Prélever 100 µl (1,5 x 10^5) de protoplastes avec une pipette dont le cône a été sectionné et les placer dans un nouveau tube de 15 ml à bout arrondi
- Ajouter 10 µl (10 µg) d'ADN purifié et mélanger soigneusement
- Ajouter 110 µl de polyéthylène-glycol (PEG) et mélanger soigneusement
- Incuber le mélange de transfection pendant 30 min
- Ajouter soigneusement 15 ml de solution W5 (au goutte à goutte pour les premiers millilitres)
- Centrifuger à 100g pendant 2 min
- Jeter le surnageant et remettre délicatement en suspension les protoplastes dans 1 ml de solution WI
- Incuber la solution pendant 24h à 23°C dans l'obscurité en inclinant le tube (augmentation de la surface solution/air)

Solutions

- **Solution de digestion :** 0,25% p/v Macerozyme Onozuka R-10, 1% p/v Cellulase Onozuka R-10 (Yakult, Japon), 0,4 M mannitol, 20 mM acide 4-morpholineethanesulfonique (MES) pH 5,7, 20 mM KCl, 10 mM $CaCl_2$, 5 mM β-mercaptoéthanol, 0,1% d'albumine de sérum bovin (BSA). Chauffer à 55°C pendant 10 min et laisser refroidir avant l'ajout du $CaCl_2$, du β-mercaptoéthanol et de la BSA.
- **Solution W5** : 125 mM $CaCl_2$, 154 mM NaCl, 5 mM KCl, 2 mM MES pH 5,7
- **Solution WI** *(Washing and Incubation)*: 0,5 M mannitol, 0,5 M KCl, 4 mM MES pH 5,7
- **Solution MaMg** : 0,4 M mannitol, 15 mM $MgCl_2$, 4 mM MES pH 5,7
- **Solution PEG** : 40% v/v PEG-4000 (Fluka # 81240), 0,8 M mannitol, 1 M $CaCl_2$, 1 M KCl

Figure 22 : Protocole de transfection des protoplastes d'*Arabidopsis thaliana*

Un site de restriction de l'endonucléase I-SceI a été placé entre le double promoteur 35S et la séquence codante d'H2B-YFP, et un second en aval de son terminateur NOS (**Figure 21 A**).

En absence de clivage, la protéine de fusion H2B-YFP est exprimée et confère une fluorescence jaune nucléaire à la cellule (**Figure 21 B**). La double digestion par l'enzyme I-SceI excise le fragment H2B-YFP-T_{NOS} du plasmide, ce qui empêche la production de la protéine. Les deux extrémités d'ADN ainsi générées peuvent être reliées entre elles par recombinaison non homologue (voies de jonction des extrémités) ou par recombinaison homologue (voie SSA ou SDSA) entre les deux séquences répétées situées de part et d'autre de la cassure. Dans le premier cas, le double promoteur 35S permet la production de la protéine de fusion tdTomato-CENH3 qui confère une fluorescence rouge à la cellule. Les évènements de SSA ou SDSA en revanche reconstituent le gène de fusion H2B-CFP dont la protéine est responsable d'une fluorescence cyan (**Figure 21 B**). L'efficacité relative des deux processus peut donc être quantifiée par la couleur de la fluorescence.

II. Analyses de la recombinaison en microscopie à fluorescence

La première étape a consisté en l'optimisation du protocole de transfection des protoplastes d'*Arabidopsis* par le polyéthylène glycol (PEG), après leur préparation à partir de jeunes feuilles. Les principales difficultés rencontrées ont été la mort d'une grande partie des protoplastes au cours des différentes étapes du protocole de transfection, ainsi que la variabilité de l'efficacité de transfection. Nos expériences ont mené à l'établissement d'un protocole optimisé de préparation et de transfection des protoplastes d'*Arabidopsis*, à partir du protocole de J. Sheen (Yoo *et al.*, 2007) (**Figure 22**).

Les protoplastes sauvages transformés avec le plasmide rapporteur de recombinaison circulaire (non digéré par I-SceI) ont d'abord été analysés au microscope à fluorescence. Jusqu'à 70 % d'entre eux présentent un marquage jaune nucléaire relativement homogène

Toutes les étapes sont effectuées à température ambiante.

- Prélever 100 µl de solution de protoplastes avec une pipette dont le cône a été sectionné et les placer dans un tube de 2 ml
- Ajouter 100 µl de Buffer Chopping contenant du triton à 0,6 %
- Agiter soigneusement et incuber 20 min sans agitation
- Ajouter 400 µl de solution GB et mélanger par retournement du tube
- Ajouter le marqueur d'ADN : DAPI (concentration finale : 5 µg/ml) ou iodure de propidium (concentration finale : 10 µg/ml).

Solutions

- **Buffer Chopping :** 45 mM $MgCl_2$, 30 mM Na citrate, 20 mM MOPS pH 7.0, 0,6 % p/v Triton X-100, 1% PVP, 1% d'albumine de sérum bovin (BSA)
- **Solution GB :** 45 mM $MgCl_2$, 30 mM Na citrate, 20 mM MOPS pH 7.0, 0,1 % p/v Triton X-100, 1% BSA

Figure 23 : Protocole d'isolation des noyaux de protoplastes d'*Arabidopsis thaliana*

(colocalisation de la fluorescence YFP et du marquage DAPI), 24h après transfection. Comme attendu, aucun protoplaste ne présente de marquage rouge ou cyan. La transfection simultanée des protoplastes avec le plasmide rapporteur de recombinaison et un plasmide d'expression de l'enzyme I-SceI (sous le contrôle d'un promoteur 35S) réduit par deux la quantité de protoplastes fluorescents en jaune (32 % de protoplastes YFP+). Des protoplastes présentant un marquage cyan (CFP+) ou rouge (Tomato+) ont également été observés, mais en quantité très faible (3 et 1%, respectivement). Il est intéressant de noter que ces protoplastes sont également YFP+. Deux hypothèses peuvent être émises : plusieurs copies du plasmide sont présentes dans ces protoplastes et seulement certains d'entre eux ont subi un évènement de recombinaison et/ou des protéines H2B-YFP sont produites avant la digestion par I-SceI et persistent dans le noyau des protoplastes.

III. Analyses de la recombinaison en cytométrie en flux

Face à la faible fréquence des protoplastes CFP+ et Tomato+, une analyse par cytométrie en flux de la fluorescence des protoplastes transfectés avec le plasmide rapporteur de recombinaison a été mise au point. Les avantages du cytomètre par rapport au microscope à fluorescence sont principalement la vitesse d'acquisition et l'analyse simultanée de plusieurs paramètres (fluorescences YFP, CFP, Tomato et du marqueur d'ADN). L'observation des protoplastes au microscope a révélé que les chloroplastes gênent souvent la détection de la fluorescence (noyau situé derrière les chloroplastes par rapport à l'expérimentateur). Afin d'évaluer précisément la fluorescence nucléaire, les noyaux sont isolés à partir des protoplastes en solution, 24h après transfection. Le protocole d'isolation des noyaux utilisé a été établi à partir du protocole de D. Galbraith (Galbraith, 2009) et des recommandations de S. Brown (CNRS, Gif-sur-Yvette) (**Figure 23)**.

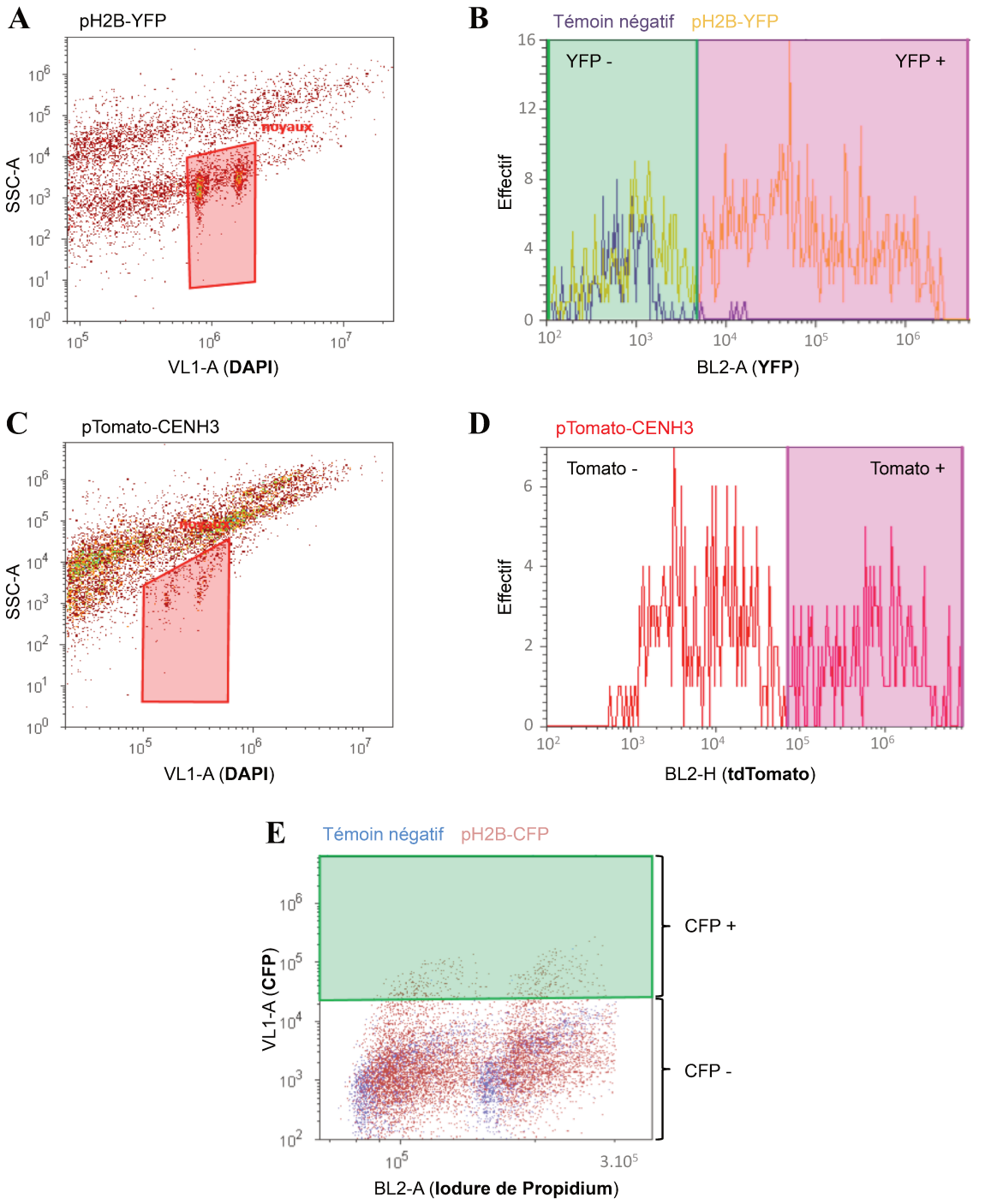


Figure 24 : Détection des protéines de fusion H2B-YFP, tdTomato-CENH3 et H2B-CFP par cytométrie en flux.

(A-B) Noyaux de protoplastes transfectés avec le plasmide pH2B-YFP et marqués au DAPI. SSC *(side scatter)* ; Rectangle rouge : noyaux (les deux populations correspondent aux noyaux 4C et 8C, C étant la quantité d'ADN d'une cellule haploïde en G1). (B) Courbe bleue : témoin négatif (noyaux de protoplastes ayant subi le protocole de transfection mais sans ajout d'ADN plasmidique) ; courbe jaune/orange : noyaux de protoplastes transfectés avec pH2B-YFP.

(C-D) Noyaux de protoplastes transfectés avec le plasmide pTomato-CENH3 et marqués au DAPI.

(E) Noyaux de protoplastes transfectés avec le plasmide pH2B-CFP et marqués à l'iodure de propidium.

La quantification de la fluorescence nucléaire nécessite au préalable l'identification de la population de noyaux grâce à un marquage de l'ADN. Afin de limiter les chevauchements des spectres d'excitation et d'émission du marqueur d'ADN et des protéines fluorescentes étudiées, la stratégie suivante a été adoptée. Les noyaux des protoplastes sont isolés 24h après transfection puis séparés en deux lots : le premier est marqué au DAPI et permettra la détection de la fluorescence YFP et Tomato (évènements de jonction des extrémités), le second est marqué à l'Iodure de Propidium pour la détection de la fluorescence CFP (évènements de recombinaison homologue).

La détection de chacune des protéines de fusion par le cytomètre en flux a d'abord été vérifiée. Pour cela, des protoplastes sauvages ont été transfectés avec les plasmides d'expression des protéines de fusion individuelles : pH2B-YFP, pTomato-CENH3 ou pH2B-CFP. L'analyse par cytométrie en flux a permis dans chacun des cas de mettre en évidence deux populations de noyaux selon leur niveau de fluorescence, démontrant que le cytomètre détecte les noyaux exprimant chacune des protéines de fusion (**Figure 24**). Avec le plasmide rapporteur de recombinaison, des résultats similaires à ceux observés par microscopie ont été obtenus : la digestion *in vivo* du plasmide par l'enzyme I-SceI n'aboutit qu'à un très faible nombre de noyaux Tomato+ ou CFP+ (< 3%). Afin de garantir la digestion du plasmide par I-SceI, cette étape a été réalisée *in vitro* et vérifiée par électrophorèse, avant la transfection des protoplastes. Pourtant, ceci ne favorise pas la reconstitution des gènes de fusion P35S-tdTomato-CENH3 et P35S-H2B-CFP par recombinaison puisque la proportion des noyaux marqués reste inférieure à 3%. La quantification de l'efficacité relative des deux modes de réparation dans les cellules sauvages est donc compromise par la rareté de ces évènements dans notre système d'étude.

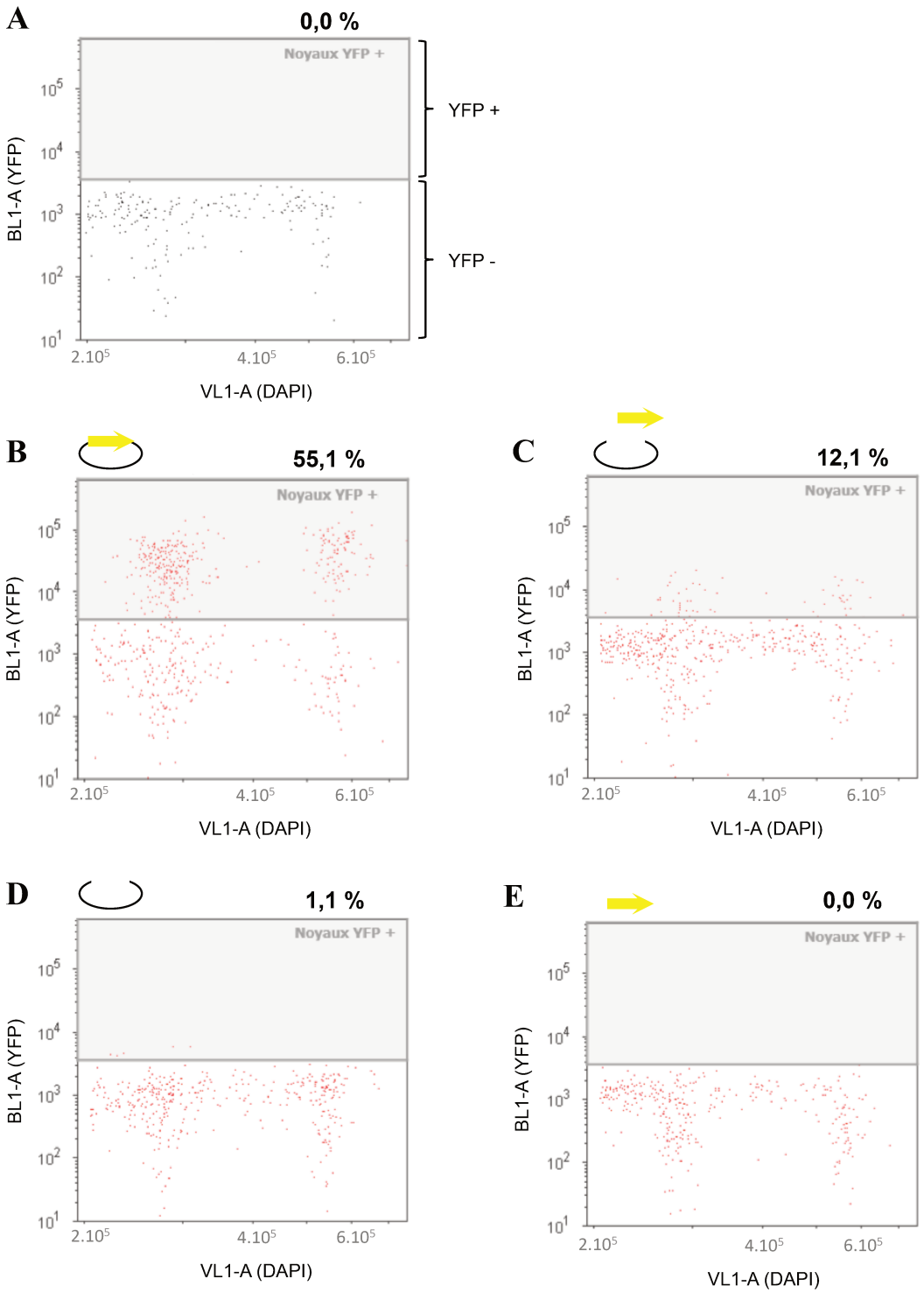


Figure 25 : Détection de la fluorescence YFP de protoplastes transfectés avec le plasmide rapporteur de recombinaison par cytométrie en flux.

Des protoplastes issus de jeunes feuilles d'*Arabidopsis* sauvages (écotype Columbia) sont soit non transfectés (A), soit transfectés avec le plasmide rapporteur de recombinaison circulaire (B) ou préalablement digéré par I-SceI (C) (cf protocole figure 22). Deux autres lots de protoplastes sont transfectés soit avec le plasmide sans le gène de fusion H2B-YFP (D), soit uniquement le fragment H2B-YFP (flèche jaune) (E). Les noyaux des protoplastes sont extraits puis marqués avec du DAPI (5 µg/ml). Les chiffres en gras correspondent aux pourcentages de noyaux fluorescents YFP+.

IV. Un système rapporteur de l'efficacité de jonction d'extrémités d'ADN

Bien que la reconstitution du gène P35S-tdTomato-CENH3 après digestion *in vitro* du plasmide soit un évènement rare, la réinsertion du fragment H2B-YFP semble plus fréquente. En effet, la transfection du produit de digestion (plasmide linéaire + fragment H2B-YFP) aboutit à plus de 12 % de noyaux YFP+ (**Figure 25 C**) - contre environ 1% de noyaux Tomato+. L'efficacité de la digestion *in vitro* par I-SceI a été vérifiée par transfection du plasmide linéaire (sans H2B-YFP) seul ou du fragment H2B-YFP seul après extraction des deux produits de digestion sur gel (**Figure 25 D-E**). Comme la recircularisation du plasmide, la réintégration du fragment excisé nécessite au minimum un évènement de jonction des extrémités d'ADN (entre le double promoteur 35S et la séquence codante d'HB2-YFP). Deux hypothèses peuvent être émises pour expliquer la surprenante préférence du substrat pour la recombinaison bimoléculaire (réintégration du fragment excisé) plutôt que unimoléculaire (recircularisation). Il est d'abord probable que la concentration de plasmides linéaires et d'inserts dans les noyaux des protoplastes transfectés soit très élevée, donc que la probabilité de rencontre entre ces deux molécules soit importante. De plus, les deux sites de restriction de l'enzyme I-SceI dans le plasmide utilisé sont en orientation inversée ; la recircularisation du plasmide nécessite donc une étape de maturation des extrémités d'ADN avant ligature. En revanche, les extrémités du plasmide linéaire et de l'insert sont compatibles (complémentarité des extrémités 3' sortantes), ce qui pourrait favoriser la réintégration du fragment H2B-YPF par simple ligature. Un variant du plasmide rapporteur de recombinaison portant les deux sites de restriction d'I-SceI dans la même orientation a également été construit au laboratoire. La digestion *in vitro* puis la transfection de ce plasmide dans des protoplastes sauvages permettrait de tester cette hypothèse.

Au même titre que la recircularisation du plasmide linéaire, la réintégration du fragment H2B-YFP constitue (au minimum) un évènement de jonction d'extrémités d'ADN. Suite à la transfection du plasmide digéré *in vitro*, les noyaux Tomato+ mais aussi YFP+ sont donc des marqueurs d'évènements de recombinaison non homologue. Ainsi, environ 13 % des protoplastes ont subi au moins un évènement de recombinaison non homologue (1 % de noyaux Tomato+ et 12 % YFP+), contre 3 % ayant subi un évènement de recombinaison homologue (CFP+). Ces premières analyses suggèrent donc que dans les cellules sauvages, les voies de jonction des extrémités d'ADN sont plus efficaces que les voies de recombinaison homologue pour la réparation de CDB induites par I-SceI dans notre système d'étude. La quantification précise des évènements de recombinaison homologue reste cependant délicate à cause de la faible fréquence de ces évènements (< 3%).

L'ensemble de ces travaux a permis de développer un outil permettant d'évaluer la capacité des cellules végétales à réaliser la jonction d'extrémités d'ADN *in vivo*, reflétant l'efficacité des voies de recombinaison non homologue. Cet outil, maintenant disponible au laboratoire, a été utilisé pour tester le rôle de la protéine XRCC2 dans la jonction des extrémités d'ADN (Résultats, Chapitre I, Résultats complémentaires, II). Il ouvre de plus la possibilité de caractériser les voies de recombinaison non homologue en étudiant notamment les substrats préférentiels de ces différentes voies ainsi que le type de jonctions qu'elles produisent au cours de la réparation. Dans ce sens, plusieurs types d'extrémités peuvent être générées : extrémités franches (*via* HpaI, **Figure 21 A**), cohésives (*via* I-SceI), compatibles ou non-compatibles (selon l'orientation des sites de restriction d'I-SceI). La modification chimique des extrémités (absence de phosphate en 5' ou de groupement OH en 3', sucres ou bases modifiées...) peut également être envisagée.

Discussion générale & Perspectives

Mes travaux de thèse se sont principalement focalisés sur l'étude des acteurs et des interactions entre les voies de recombinaison homologue chez la plante modèle *Arabidopsis thaliana*. Dans les cellules somatiques, les deux voies majeures de réparation des CDB par recombinaison homologue sont la voie SSA *(single strand annealing)* et la voie SDSA *(synthesis-dependant strand-annealing)* (Pâques & Haber, 1999 ; Symington, 2002b ; Ira *et al.*, 2006). Dans un souci de simplification du modèle d'étude - par rapport à l'éventail de lésions générées par l'irradiation - mon travail a porté sur l'analyse de la réparation d'une CDB unique et localisée, induite par la méganucléase I-SceI.

Notre intérêt s'est d'abord porté sur trois acteurs de la recombinaison homologue, XRCC2, RAD51B et RAD51D, des paralogues de RAD51. Chez les animaux comme chez les plantes, ces protéines jouent le rôle de médiateur de l'activité de la recombinase RAD51 (pour revue, voir Suwaki *et al.*, 2011). Bien que leurs rôles exacts restent à identifier, il a été montré qu'ils agissent au cours de plusieurs étapes des voies de recombinaison homologue dépendantes de RAD51 : la formation et la stabilisation du nucléofilament RAD51-ADNsb (Takata *et al.*, 2000 ; Takata *et al.*, 2001 ; Chun *et al.*, 2013 ; Da Ines *et al.*, 2013a), ainsi que l'échange de brin catalysé par RAD51 (Sigurdsson *et al.*, 2001).

Grâce à l'induction d'une CDB dans le locus DGU.US (Orel *et al.*, 2003) par l'enzyme I-SceI, nous avons montré que l'efficacité de reconstitution du gène GUS par recombinaison est nettement diminuée dans les plantes *xrcc2* par rapport aux plantes sauvages (**Article 1, figure 1**). Etant donné que cette construction est rapportrice d'évènements de recombinaison homologue indépendants de RAD51 (Roth *et al.*, 2012), ces données démontrent que XRCC2 est impliqué dans la voie SSA. Afin de vérifier ce résultat totalement inattendu à l'échelle moléculaire, nous avons développé une analyse par Southern blot permettant de visualiser et de quantifier précisément les produits de la recombinaison. Bien que le substrat DGU.US soit

largement utilisé dans la littérature, cette analyse n'avait à notre connaissance jamais été menée sur plante entière. La confirmation du rôle de XRCC2 dans la voie SSA (**Article 1, figure 2**) nous a conduit à rechercher les fonctions potentielles de RAD51B et RAD51D, deux autres paralogues de RAD51. Puisque l'efficacité du SSA est clairement réduite dans les mutants *rad51b* et *rad51d* par rapport à des plantes sauvages, ces deux protéines sont également impliquées dans la voie SSA (**Article 1, figures 5 et 6**). L'analyse du double mutant *xrcc2 rad51b* et du triple mutant *xrcc2 rad51b rad51d* a de plus révélé que ces trois paralogues de RAD51 jouent des fonctions non-épistatiques (au moins pour XRCC2 et RAD51B) dans cette voie de recombinaison indépendante de RAD51 (**Article 1, figure 5**). Afin de confirmer que le rôle de la protéine XRCC2 dans le SSA ne dépend pas de RAD51, le double mutant *xrcc2 rad51* a été généré. Des résultats similaires au simple mutant *xrcc2* ont été obtenus (**Figure 20**), ce qui confirme que la fonction de la protéine XRCC2 dans le SSA est bien indépendante de RAD51. A notre connaissance, ces travaux constituent la première démonstration de l'existence d'une fonction d'un paralogue de RAD51 dans la recombinaison indépendante de RAD51, chez les Eucaryotes.

Quels sont les rôles de XRCC2, RAD51B et RAD51D dans la voie SSA ? Des études *in vitro* ont montré que le complexe BCDX2 présente une grande affinité pour les structures d'ADN branchées, telles que les structures en Y issues de l'hybridation des séquences répétées au cours du SSA (Yokoyama *et al.*, 2004). Les mêmes auteurs ont démontré que ce complexe est capable de catalyser l'hybridation de fragments d'ADNsb *in vitro*. Ceci suggère très fortement que ces paralogues soient impliqués dans l'étape d'hybridation des séquences complémentaires situées de part et d'autre de la cassure. En revanche, l'absence de RAD51C (un membre du complexe BCDX2) n'induit qu'une très faible diminution de l'efficacité du SSA chez *Arabidopsis* (Roth *et al.*, 2012). La différence de sévérité des phénotypes des simples mutants (*xrcc2*, *rad51b*, *rad 51c* et *rad51d*), ainsi que l'existence de fonctions non-

épistatiques suggèrent des rôles individuels de ces protéines, plutôt que sous la forme d'un complexe. La vérification de ces hypothèses nécessite d'entreprendre des études biochimiques des paralogues de RAD51 d'*Arabidopsis*. Dans ce sens, nous envisageons de produire ces protéines dans un système bactérien puis de réaliser des tests d'activité *in vitro* des protéines individuelles ou sous forme de complexe.

Une alternative à ce rôle direct dans l'étape d'hybridation des séquences répétées est une action au cours de la résection des extrémités de la cassure. Cette étape est en effet commune aux différentes voies dans lesquelles XRCC2, RAD51B et RAD51D interviennent : les voies de recombinaison homologue dépendantes de RAD51 (pour revue, voir Suwaki *et al.*, 2011) et la voie SSA (Article 1). De plus, il a été montré que ces protéines agissent en amont du recrutement de RAD51 (Chun *et al.*, 2013), soit à une étape très précoce de la recombinaison, ce qui serait compatible avec un rôle dans la résection. Il est possible de suivre la résection des extrémités des CDB *in vivo* grâce à un marquage immunohistochimique de la protéine RPA, qui se lie à l'ADN simple brin (Osman *et al.*, 2009). Ce type d'analyse nécessite de connaître la cinétique de formation puis de réparation de la CDB dans une cellule sauvage. Dans cette optique, nous avons quantifié les foci du variant d'histone H2AX phosphorylé (γH2AX), un marqueur spécifique des CDB, à différents temps après l'induction de l'expression d'I-SceI dans des plantules d'*Arabidopsis*. Les résultats préliminaires sont encourageants mais la rapidité de la réparation des CDB (Charbonnel *et al.*, 2011) et l'asynchronie des évènements dans les différentes cellules compliquent actuellement l'analyse.

La seconde partie de mes travaux de thèse s'est focalisée sur l'analyse des fonctions du complexe XPF-ERCC1 dans les voies de recombinaison homologue (RH). Ce complexe présente une activité endonucléasique spécifique de la structure de l'ADN, responsable du clivage des extrémités d'ADN non homologues des intermédiaires de recombinaison (Fishman-Lobell & Haber, 1992 ; Bardwell *et al.*, 1994 ; Ivanov & Haber, 1995 ; Adair, 2000 ; Sargent *et al.*, 2000 ; Dubest *et al.*, 2002 ; Dubest *et al.*, 2004 ; Al-Minawi *et al.*, 2008). Chez *Arabidopsis*, l'inactivation de XPF ou ERCC1 inhibe d'une part la voie SSA - quand celle-ci implique le clivage de longues extrémités 3' non homologues - et d'autre part, les voies de RH dépendantes de RAD51 (Dubest *et al.*, 2002 ; Dubest *et al.*, 2004). Cependant, en utilisant les loci rapporteurs de recombinaison DGU.US et IU.GUS (Orel *et al.*, 2003), nous avons observé que l'efficacité de reconstruction du gène GUS n'est pas réduite dans les plantes mutantes *xpf* (**Article 2, figures 2 et 4**). Contrairement au substrat U'G'.US utilisé par Dubest *et al.* (2004), la réparation de ces loci par RH nécessite le clivage d'extrémités non homologues de petite taille (< 21 nucléotides) (**Article 2, figure supplémentaire 1**). Grâce à un système plasmidique analogue à celui utilisé par Dubest *et al.* (2002, 2004) - mais produisant des extrémités non homologues de petite taille - nous avons démontré que le complexe XPF-ERCC1 n'est pas nécessaire au clivage des courtes extrémités 3' sortantes au cours de la RH chez *Arabidopsis* (**Article 2, figure 1**). Ces résultats sont parfaitement en accord avec les expériences menées chez la levure et les Mammifères. En effet, la réparation par RH d'une CDB dont les extrémités contiennent des séquences non homologues de moins de 30 nucléotides est indépendante du complexe Rad1-Rad10 (l'homologue de XPF-ERCC1) chez *S. cerevisiae* (Pâques & Haber, 1997). Dans les cellules CHO, les extrémités non homologues dont la taille est inférieure à 18 nucléotides sont efficacement éliminées en absence de la protéine ERCC1 (Adair, 2000 ; Sargent *et al.*, 2000). Il a de plus été rapporté

que la protéine ERCC1 humaine présente une faible affinité pour les extrémités d'ADNsb de moins de 15 nucléotides (Tsodikov *et al.*, 2005).

Afin de poursuivre cette analyse, il serait d'abord intéressant de préciser la taille limite à partir de laquelle le complexe XPF-ERCC1 est requis pour l'élimination des séquences non homologues. Pour cela, différentes digestions enzymatiques des plasmides pGF et pFP (Article 2) permettraient la génération de substrats avec des séquences non homologues de taille variable. L'efficacité de la recombinaison pourra être testée en protoplastes sauvages ou mutants pour XPF (ou ERCC1), selon la méthode décrite dans l'article 2. L'initiation de la synthèse d'ADN puis la ligature des brins au cours des voies SSA et SDSA nécessitent l'élimination de toutes les extrémités non homologues, quelle que soit leur longueur. Puisque l'élimination des courtes extrémités est indépendante du complexe XPF-ERCC1, quelle activité enzymatique réalise cette fonction ? Chez *S. cerevisiae*, les extrémités de 10 ou 20 nucléotides sont dégradées par un mécanisme qui dépend au moins en partie de l'activité de relecture 3'-5' de la polymérase δ (Pâques & Haber, 1997). Sachant qu'un homologue de la polymérase δ a été identifié chez *Arabidopsis* (Schuermann *et al.*, 2009), cette enzyme pourrait être un bon candidat.

Dans un contexte où XPF-ERCC1 n'est pas requis à la recombinaison SDSA (taille des extrémités non homologues inférieure à 18 nucléotides), une augmentation inattendue de la recombinaison au locus IU.GUS a été observée dans les plantes mutantes *xpf* (**Article 2, figure 4, tableau 2**). La complémentation de ces mutants avec la protéine de fusion XPF-GFP a permis de confirmer que l'augmentation de l'efficacité du SDSA est strictement liée à l'absence de la protéine XPF (**Article 2, figure 5**). XPF, et probablement le complexe XPF-ERCC1, joue donc un rôle inhibiteur sur la recombinaison SDSA. Ce résultat rappelle le rôle anti-recombinogène de ce complexe dans la protection des télomères courts chez *Arabidopsis* (Vannier *et al.*, 2009) et les Mammifères (Zhu *et al.*, 2003). En effet l'inactivation de XPF ou

ERCC1 dans un contexte de télomères déprotégés stimule fortement l'instabilité chromosomique. Ceci semble être le résultat de l'invasion d'extrémités 3' télomériques dans des régions interstitielles de répétitions télomériques dégénérées. Le complexe XPF-ERCC1 serait impliqué dans le clivage de ces extrémités 3' sortantes et inhiberait ainsi l'invasion de séquences ectopiques par les extrémités télomériques (Zhu *et al.*, 2003 ; Vannier *et al.*, 2009).

Nous proposons une explication analogue à l'effet recombinogène observé au locus IU.GUS en absence d'XPF. Afin de vérifier que l'activité nucléase d'XPF est spécifiquement responsable de l'inhibition de la recombinaison SDSA, nous avons construit la protéine XPF_{D757A}-GFP mutée dans le site actif d'XPF (par analogie avec la protéine humaine (Enzlin & Schärer, 2002)). Contrairement à la protéine fonctionnelle (XPF-GFP), la présence d'XPF_{D757A}-GFP ne réduit pas l'efficacité du SDSA dans les plantes *xpf* (**Article 2, figure 5**). Le rôle inhibiteur d'XPF sur la recombinaison SDSA est donc dépendant de sa capacité à cliver l'ADN.

Des études biochimiques ont révélé que le complexe XPF-ERCC1 est capable de cliver différents types de substrats *in vitro*, et notamment les extrémités d'ADNsb 3' sortantes de fragments d'ADNdb ou de molécules d'ADN branchées (De Laat *et al.*, 1998a). Une première hypothèse pour expliquer le rôle inhibiteur du complexe sur la recombinaison SDSA est qu'il clive les extrémités d'ADN 3' sortantes générées au cours de la résection. Cependant, ce clivage aurait un effet hypo-recombinogène général en limitant à la fois les voies SSA et SDSA, puisque les deux processus nécessitent la présence d'extrémités d'ADNsb. Une augmentation de l'efficacité de la recombinaison DGU.US -> GUS devrait donc être observée en absence de la protéine XPF (réparation par SSA et/ou SDSA), mais aussi en absence d'XPF et de RAD51 fonctionnel (réparation par SSA). Puisque l'efficacité de la réparation est similaire dans les plantes sauvages et les plantes *xpf* RAD51-GFP (**Article 2, figure 3**), XPF n'a pas d'effet sur la voie SSA (dans le cas où les extrémités non homologues sont courtes), ce

qui infirme cette hypothèse. Selon le modèle de Agmon *et al.*, la recherche d'homologie puis l'appariement de quelques bases (au niveau de microhomologies) entre les deux extrémités d'ADNsb de la cassure forme une structure d'ADN branchée avec des extrémités d'ADN 3' sortantes (Agmon *et al.*, 2009). Une seconde hypothèse serait que le complexe XPF-ERCC1 reconnaisse ces structures et clive les extrémités 3' sortantes, limitant ainsi la voie SDSA (puisque les extrémités réalisant l'invasion ont été éliminées), sans affecter *a priori* la voie SSA.

Dans ce contexte, nous proposons un modèle expliquant le phénotype hyper-recombinogène des plantes mutantes *xpf* par la modulation de la balance entre les voies de RH (associées à la reconstitution du gène GUS) et la voie MMEJ (non productive) (**Article 2, figure 6**). Les CDB induites par I-SceI dans le substrat DGU.US seraient réparées soit par la voie SSA, soit par la voie MMEJ suite à l'appariement de courtes régions (microhomologies) entre les deux extrémités simple brin de la cassure. En absence de la protéine XPF, le clivage des longues extrémités 3' sortantes nécessaire à la réparation de la CDB par MMEJ est inhibé. Ces intermédiaires de recombinaison seraient alors redirigés vers la voie SDSA *via* l'invasion puis la copie d'une séquence homologue par l'une des extrémités non clivées. Ceci explique d'une part l'augmentation globale du nombre de spots recombinants observée dans les mutants *xpf* par rapport aux plantes sauvages (**Article 2, figures 2 et 3, tableau 1**), et d'autre part que cette augmentation soit dépendante de RAD51 (**Article 2, figure 3**). Dans le cas du substrat IU.GUS, les CDB induites par I-SceI seraient prises en charge soit par la voie SDSA, soit par la voie MMEJ (**Article 2, figure 6**). Comme précédemment, l'augmentation du nombre de spots recombinants en absence de la protéine XPF serait liée à la redirection des intermédiaires de recombinaison présentant de longues extrémités d'ADN 3' sortantes, vers la voie SDSA.

Afin de tester ce modèle, nous envisageons de séquencer les produits de réparation des substrats DGU.US et IU.GUS dans des plantes sauvages et des plantes *xpf* après induction de l'expression de l'enzyme I-SceI. La quantification relative des différents types de produits permettra d'abord de vérifier l'importance de la voie MMEJ dans la réparation des CDB induites par I-SceI et de confirmer à l'échelle moléculaire la contribution relative des différentes voies mises en jeu.

L'ensemble de ces travaux a permis de préciser les fonctions des protéines XRCC2, RAD51B, RAD51D, XPF et ERCC1 dans les voies de recombinaison homologue en contexte somatique chez *Arabidopsis thaliana*. Nous avons démontré un rôle inattendu des protéines XRCC2, RAD51B et RAD51D dans la voie SSA indépendante de RAD51 (Article 1). Ces travaux ont mis en évidence pour la première fois que certains paralogues de RAD51 possèdent également des fonctions indépendantes de la recombinase. Nous avons de plus démontré que le complexe XPF-ERCC1 joue un rôle inhibiteur sur la voie de recombinaison SDSA (Article 2). Grâce à son activité nucléase, celui-ci cliverait les extrémités d'ADN 3' sortantes des intermédiaires de recombinaison, limitant ainsi la recherche d'homologie par les extrémités.

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